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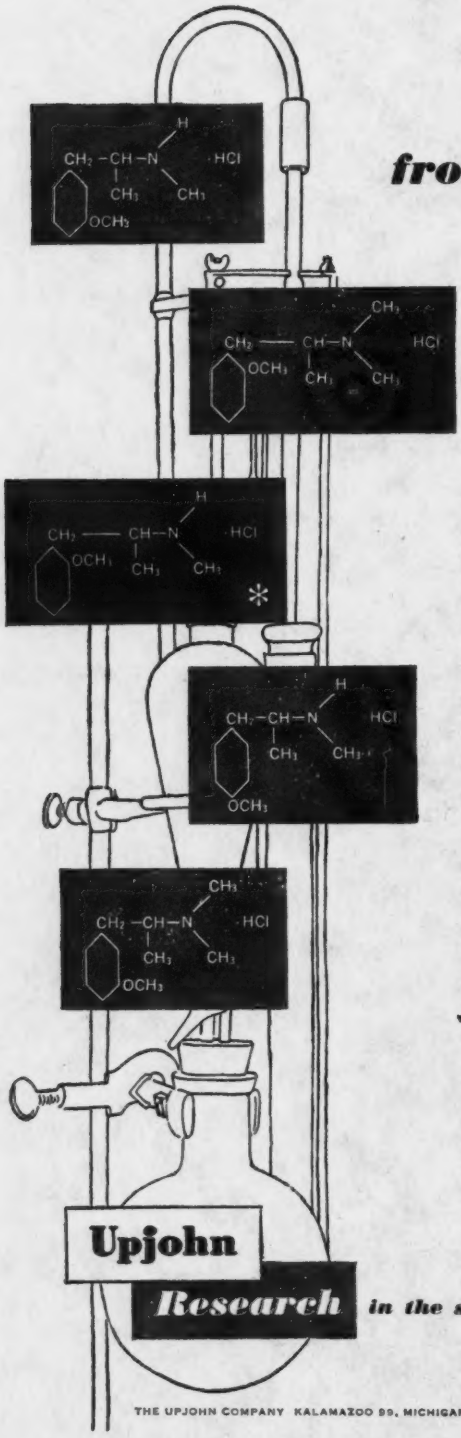

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ENDOCRINOLOGY

VOLUME 46 MARCH, 1950 NUMBER 3

DISAPPEARANCE RATE OF ADRENOCORTICOTROPIC HORMONE FROM RAT'S PLASMA AFTER INTRAVENOUS INJECTION[1]

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BERKELEY, CALIFORNIA

THERE is reason to suppose that the adrenocorticotropic hormone disappears rapidly from the blood stream but there are few or no determinations of the actual rate of disappearance.

TABLE 1. STANDARDIZATION OF PURE ADRENOCORTICOTROPIC HORMONE BY THE ASCORBIC ACID DEPLETION METHOD

Dose ACTH micrograms/100 gm. B. W.	Ascorbic acid depletion mg./100 gm. fresh adrenal
0	6 ± 8.3 (27)*
0.2	43.3 ± 7.3 (23)
0.5	59.9 ± 3.2 (10)
1.0	73.0 ± 6.4 (13)
2.0	100.5 ± 6.3 (11)
3	100.7 ± 11.6 (12)
5	115.8 ± 6.9 (13)
10	145.4 ± 4.8 (8)
20	149.3 ± 9.7 (10)
50	194.8 ± 6.9 (5)

* Mean in mg. per 100 g. fresh tissue ± standard error; number of animals in parentheses.

PROCEDURE

Male rats, of the Long-Evans strain, weighing 440 to 500 gms., were anesthetized with ether and injected intravenously, via the tail vein, with 5 m. of pure adrenocorticotropic hormone (ACTH). The hormone was prepared by the method of Li *et al.* (1943). After

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[1] Aided by grants from the U. S. Public Health Grant-RG-409 and the Research Board of the University of California, Berkeley, California.

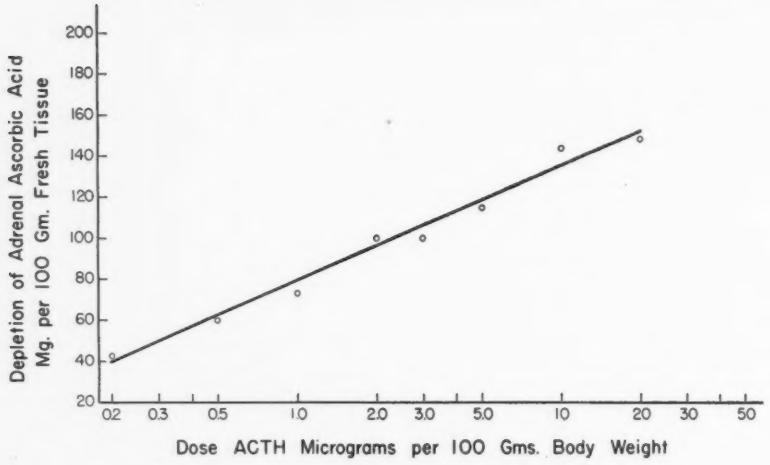

FIG. 1. Depletion of adrenal ascorbic acid following intravenous administration of ACTH.

various time intervals, the animals were reanesthetized with ether and exsanguinated, blood being withdrawn from the inferior vena cava with heparin as an anti-coagulant. The plasma was separated by centrifugation and immediately assayed in doses of 0.5 cc. per 100 gm. body weight of assay animal, for the content of ACTH, by the adrenal ascorbic acid depletion method, as reported by Sayers *et al.* (1948). A standard curve obtained with this method using pure ACTH is

TABLE 2. DISAPPEARANCE OF INTRAVENOUSLY INJECTED ACTH IN RATS AS INDICATED BY THE ASCORBIC ACID DEPLETION TEST

Each rat except the first (*) received 5 mg. of the hormone

Body wt.	Time of bleeding after injection	Ascorbic acid	Estimate of concentration of ACTH, micrograms per cc. plasma	
			Mean	Range§
g.	min.	mg.	micrograms/cc.	micrograms/cc.
520*	10	11 ± 11.1 (6)†	0	0
500	1	133 ± 18.9 (4)	18.0	8–40
440	1‡	120 ± 25.7 (4)	24.0	8–52
448	1	142 ± 16.5 (4)	26.0	11–56
452	6	119 ± 28.4 (4)	10.0	3.6–34
452	9	100 ± 8.0 (5)	4.8	3.4– 6.2
448	17	85 ± 34.7 (4)	2.6	0.6–11.2
465	25	61 ± 23.9 (3)	1.0	0.4– 2.6
500	30	44 ± 16.5 (5)	0.48	0.2– 0.8

* This rat received plasma from a rat injected with 5 mg. of human plasma protein.

† Mean ± standard error; in parentheses are the number of rats used for ascorbic acid determination.

‡ Plasma diluted 1:1 with saline prior to assay.

§ Based on std. error.

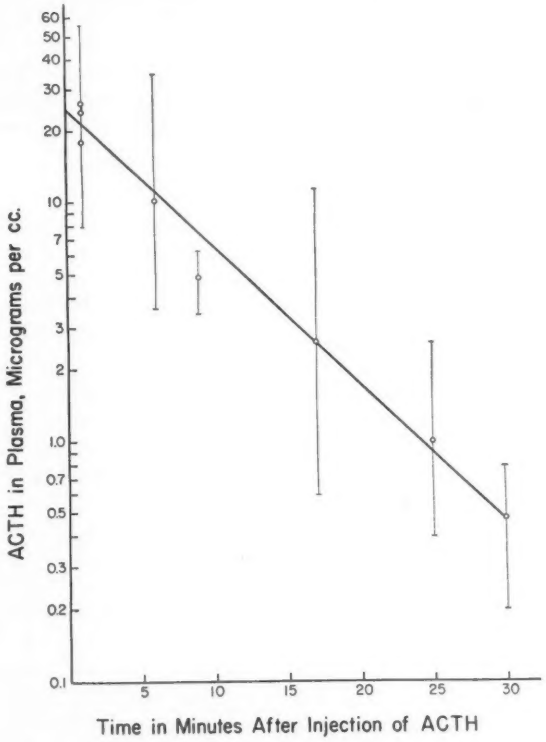

FIG. 2. The disappearance of intravenously administered ACTH from the plasma of the rat.[2]

presented in Table 1 and Fig. 1. It was found that 0.2 microgram of ACTH per 100 gm. body weight of assay animal produced a significant depletion of the adrenal ascorbic acid, and that most satisfactory working range of the curve lay between 0.2 and 10 micrograms ACTH.

RESULTS

The results of the assays of rat plasma at various time intervals after the injection of ACTH are presented in Table 2 and Fig. 2. Plasma from an animal injected with 5 mg. of human plasma protein, as a control, contained no measurable ACTH activity. Blood was withdrawn from 3 animals within 1 minute of the injection, and the level of ACTH varied from 18 to 26 micrograms per cc of plasma. There after the plasma leve of ACTH fell in a logarithmic fashion over

[2] Multiple determinations of adrenal ascorbic acid (the range is indicated by the vertical lines) were made on the effect of each serum; the circles used for the graph indicate the mean for each animal.

the period of the study. The half-time of the disappearance of ACTH in this study is $5\frac{1}{2}$ minutes. The extrapolated value of the level of ACTH at zero time is 24 micrograms per cc. plasma. The range of error in the determinations is indicated by the standard error of each assay, suggesting that the variation of each mean may be from $-50\%+100\%$

COMMENTS

It should be pointed out at the onset that the number of assay animals used is not sufficient for careful quantitative determination of the level of ACTH in the plasma. On the other hand, the data obtained strongly suggest that the level of ACTH falls in a logarithmic fashion against time, and that the rate of disappearance is rather rapid. From the ACTH level at zero time, we may obtain some indication of the immediate removal of ACTH from the plasma. Since 5000 micrograms were injected into a 500 gm. animal, and since the plasma volume of the animal is about 2.4% of the body weight (Berlin, *et al.*, 1949), the expected concentration of ACTH in the plasma would be approximately 417 micrograms per cc. of plasma. The extrapolated value at zero time was found to be 24 micrograms per cc. of plasma. Thus only 6% of the injected dose could be found in the plasma at zero time. Whether this represents an immediate inactivation process, an adsorption phenomenon, or the distribution of ACTH in body fluids, cannot be stated at the present time.

Sayers, *et al.* (1949) have recently reported the rapid disappearance of ACTH from the plasma of two human subjects following intravenous infusion but from the data reported it is not possible to determine the immediate disappearance phenomenon in man, because of the use of the prolonged infusion technique.

SUMMARY

The disappearance of a single intravenous injection of ACTH has been studied in rats.

The level of ACTH falls in a logarithmic fashion against time, and the half time of the disappearance of ACTH is approximately 5.5 minutes.

From the extrapolated level of ACTH at zero time it is estimated that only 6% of an injected dose of ACTH could be found in the plasma immediately following injection.

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FUNCTIONAL INTRASPLENIC OVARIAN TRANSPLANTS IN MONKEYS[1]

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THE INACTIVATION of estrogens by the liver has been generally assumed for animals of all species although few species have been investigated with this question in view. Zondek (1934) first demonstrated that rat's liver destroyed the normally occurring estrogens and numerous investigators have extended this observation to other species of rodents. And, conversely, Talbot (1939) by chemically destroying liver function showed a subsequent rise in circulating estrogens. However, few experiments have been conducted on the hepatic inactivation of estrogens by primates. Twombly and Taylor (1942) observed that in 17 of 22 tests in which estradiol was incubated with human liver there was little inactivation of the hormone. The hepatic tissue from the other five human donors was about half as effective in destroying estradiol as was rat's liver. In the monkey, intrasplenic implantation of pellets of estrone and estradiol was followed by reddening of the sex-skin and alteration of the vaginal smear, as promptly and with the same intensity, as in control animals given estradiol benzoate subcutaneously (Hooker, Drill and Pfeiffer, 1947). Less direct experiments, however, have indicated that the liver does destroy estrogens in man (Glass, Edmondson and Soll, 1940; Gilder and Hoagland, 1946).

MATERIALS AND METHODS

Four immature monkeys (Macaca mulatta) weighing from 1200 to 2060 grams were ovariectomized at the time of transplantation. The ovary of a young monkey in the above weight range is shaped like a miniature lima bean measuring about 6 mm. in length and 4 mm. in width. The ovary is from 2 to 3 mm. in thickness and as the left gland was removed it was made ready for transplantation by splitting it lengthwise from the free border to the hilum. The spleen was then brought into view after sufficiently extending the midline abdominal incision cephalically. A cut was made in the splenic capsule and a probe inserted with a sweeping motion to free the capsule from the stroma. There was some bleeding following this procedure. One-half of the animal's ovary was slipped under the capsule and well beyond the entrance slit. The cut surface of the ovary then lay in contact with the

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[1] This investigation was supported by a research grant from the National Cancer Institute, U. S. Public Health Service.

pulp of the spleen while the germinal epithelium was covered by the capsule. Using a curved surgical needle, the capsule was torn in three or four places just over the ovary and because the capsule was under tension these small tears opened, exposing the germinal epithelium to the peritoneal cavity, yet holding the cut surface of the gonad in close contact with the spleen. The object of the fenestration was to encourage normal surface relations of the ovarian tissue. The above technique was developed in the first operations when transplants were made to the head of the spleen, on its abdominal surface. The site of the transplantation was later changed to the underside of the head of the spleen between the blood vessels and the cephalic border of the organ to avoid the formation of adhesions from the surface of the spleen to the abdominal peritoneum. All implants to the parietal surface of the spleen were checked for adhesions but only thread-like, non vascular adhesions were found. These were broken and checked for reformation at a later laparotomy. Transplantation to the visceral surface of the spleen avoided the possibility of hormone escape to extra-portal circulation.

Of four immature monkeys, three, M m 67, M m 71 and M m 75, received homo-transplants of the ovary to the spleen. In the fourth monkey, M m 72, the ovarian transplantation was designed to place the gonad outside the region of hepatic drainage and the site chosen was the under surface of the left rectus muscle at the level of the umbilicus. A stitch of black thread was placed alongside for identification.

Two immature males, M m 68 and M m 73, were castrated when they weighed 1310 and 2300 grams, respectively. M m 68 received the ovary from a fetus weighing 182 grams (removed at uterotomy from the parent monkey on the 113th day of gestation). The second immature male, M m 73, received the other half of the left ovary from M m 75 at the time of its homo-transplantation.

Three mature, normally menstruating monkeys weighing between 2890 and 4600 grams and of estimated age between two and one-half years and four years received homo-transplants of one half of the left ovary to the spleen as described. These operations took place on the 3rd, 5th, and 24th days of the current menstrual cycles.

Four of the immature monkeys, the two males and two of the females, received a gonadotrophin[2] after several months in the hope of supporting the transplants or activating them so that survival of the tissue could be determined at a laparotomy.

All of the animals lived in a monkey colony receiving the same care and eating the same food which has been found adequate over fifteen years to support good growth in young animals and active reproduction in the mature members. The diet was similar to a balanced human diet except that the source of protein was never meat but largely eggs and milk or vegetable proteins. Vitamin supplements of C and D were given daily and since the animals lived entirely indoors a centrally located quartz mercury vapor lamp was used.

OBSERVATIONS

In all the female monkeys receiving homologous transplants the

[2] Anteron, the gonadotrophin used, was supplied through the courtesy of Dr. Edward Henderson of Schering Corporation, Bloomfield, New Jersey.

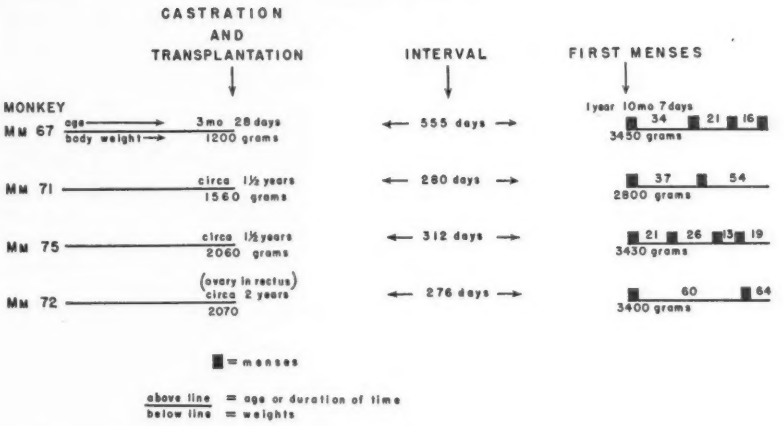

FIG. 1. Vaginal bleeding (menstrual cycles) in monkeys sexually immature when overiectomized and transplanted. One half of one ovary was placed beneath the fenestrated capsule of the spleen.

ovarian tissue persisted and became functional (Fig. 1 and Fig. 2). Ovarian activity was manifested after widely varying intervals according to the physiological maturity of the recipient (Table 1). The ovarian activity was clearly demonstrated in three different ways, by the establishment or re-establishment of cyclic uterine bleeding and cyclic vaginal desquamation and, in the third place, by development of sexual skin changes characterized by a series of concentric swellings in the adolescent animals and by the intensification of sex-skin color in the mature animals.

Three of the young monkeys, Fig. 1, purchased from a dealer

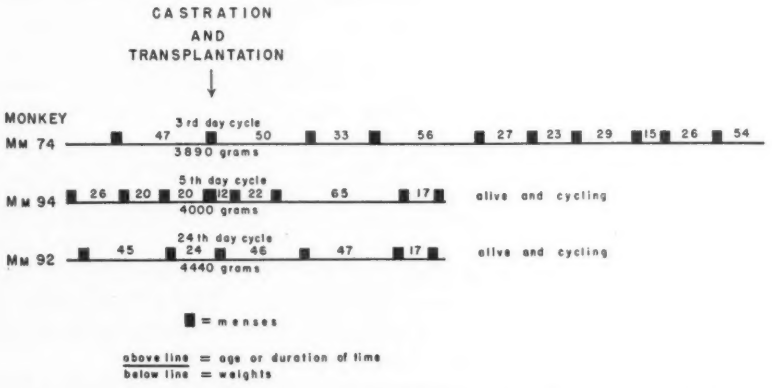

FIG. 2. Vaginal bleeding (menstrual cycles) in monkeys ovariectomized after sexual maturity and with approximately one half of one ovary transplanted beneath the fenestrated capsule of the spleen.

were of unknown age. However, their ages could be roughly estimated as $1\frac{1}{2}$ to 2 years by examination of the dentition and consideration of the body weight and length. The fourth monkey, much younger, was born in the colony and its ovary was transplanted when three months and 28 days old. One of the two young males, M m 68, was also of known age, 3 months and 24 days. The transplantations on the immature monkeys were performed in December, 1947. Exploration of the younger male after three months revealed an opacity at site of operation but no ovarian tissue could be distinguished. Pre-treatment with a gonadotrophin before further observation was decided upon

TABLE 1. SUMMARY OF TREATMENT AND OBSERVATIONS ON THE FUNCTION OF OVARIAN GRAFTS IN IMMATURE AND ADULT FEMALE MONKEYS

Monkey	Weight at transplantation	Day of cycle ovariectomized	Interval between transplantation and first menses	Weight at first menses	Number of cycles observed after transplant	Average length of cycle	Range
Immature	(grams)		(days)	(grams)		(days)	(days)
M m 67	1200	Immature	559	3450	3	34	16–66
M m 71	1560	Immature	280	2840	9	33	12–98
M m 75	2060	Immature	312	3430	9	24	13–45
M m 72*	1710	Immature	276	3400	6	45	21–64

Monkey	Weight at transplantation	Day of cycle ovariectomized	Interval following operation and before next menstruation		Number of cycles observed after transplant	Average length of cycle	Range
Mature							
M m 74	3890	3	48	—	11	34	15–56
M m 92	4600	24	46	—	3	37	17–48
M m 94	4000	5	8	—	4	29	12–60

* Ovary transplanted into rectus abdominus.

and this stimulative treatment may have been responsible for the appearance of minute cystic structures believed to be follicles seen in M m 71 and M m 75 after five months. No external evidence of function was seen at this time.

The young females of the Obstetric Monkey Colony may reach menarche as early as 1 and $\frac{1}{2}$ years but the average age of first menstruation is 2 years. They then weigh about 3500 grams. It is interesting to note (see Fig. 1) how nearly the transplanted monkeys approximated this weight and also the age, in the animal in which it was known. In this animal the ovarian transplant remained quietly in the spleen over a year and a half before the time arrived for the hypophyseal stimulation to bring it into appreciable action. The older animals reached this point after 276 to 312 day intervals, but the shortest interval was over nine months. There was no difference in external effects between the transplants within the portal system and that which was placed in the peripheral musculature.

After castration and implantation of the ovaries in three adult females, Fig. 2, there was no great interval before the menstrual cycles were re-established. The two longer post-operation cycles of

46 and 50 days were not very different from the pre-operation cycles of the same animals. Menstrual cycles in the monkey are characteristically irregular but fall around the modal length of 28 days. The beginning of the reproductive period is usually made up of relatively long cycles and repeated long cycles have been seen in animals known to be fifteen years or older. On the other hand, short cycles with rather more than normal loss of blood have also been seen in the adolescent animal. Monkey 94 was running short cycles before opera-

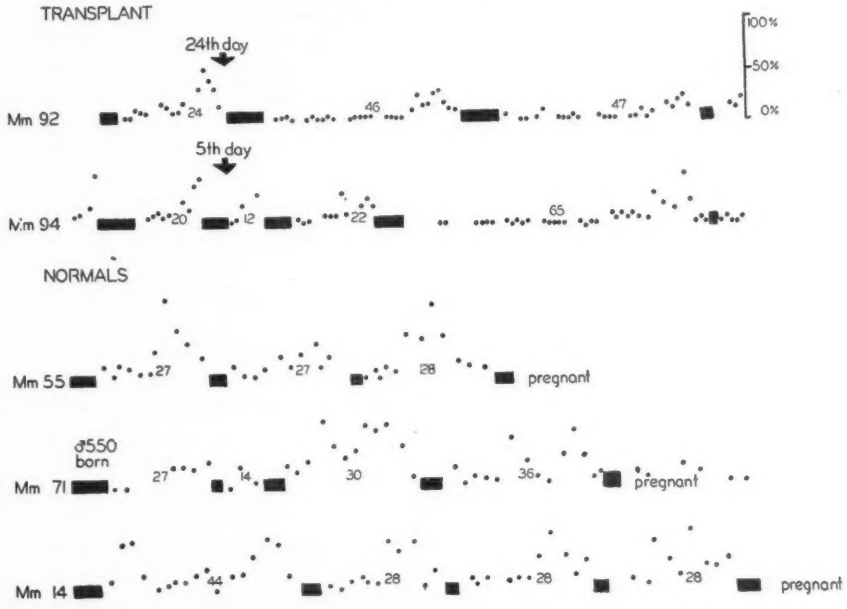

FIG. 3. Vaginal desquamation readings of the monkeys M m 92 and M m 94 after ovariectomy and transplantation of ½ of one ovary into the spleen are to be contrasted with the readings before operation and those of three normal, presumably ovulating, monkeys.

tion and the second cycle of 65 days is not remarkable because it was preceded and followed by a short cycle. In other words the menstrual histories of these three animals, in so far as they go, could not be distinguished from histories of normal monkeys. The time of operation within the cycle does not appear to influence the hypophyseal-gonad hormone balance, nor does it in any way modify the alacrity with which the gonad again begins its functioning within the new environment of the spleen.

Vaginal desquamation when assayed quantitatively as in the Hartman test (1932) reflects the estrogenic hormone levels. For example, the desquamation is meager at the time of menarche increas-

ing as the ovary increases its production of hormone in succeeding cycles and it is almost nil during the latter half of pregnancy when the estrogens are "combined."

Sedimentation readings were made almost daily for M m 92 and M m 94. Fig. 3 shows a cycle or two before transplantation as well as some typical cycles for three normal animals. Cycles just before pregnancy were selected for then the animals were presumably ovulating.

In the post operative cycles the low desquamation readings indicate the effect of the reduction of ovarian tissue. Yet the implantation of $\frac{1}{4}$ the normal amount of ovarian tissue was sufficient to respond cyclically and to bring about uterine bleeding. The longer post-operative cycles can be readily analyzed. The early phase is the one which is prolonged, very much as if this extra time is necessary for the smaller amount of gland tissue to build up to a point where events closing the cycle can be initiated.

In the immature monkeys, the perineal skin remained unchanged until just before the first bleeding when pubic swellings appeared similar in every way to the sex skin changes in the normal adolescent monkey. In the mature animals there was a lessening in intensity of the red coloration of the perineal region beginning the day following castration. It became lighter and remained so for the following six to eight weeks. The coloration is a vascular phenomenon and blanches on pressure. Thus, there was an indication of reduction of hormone effect on the blood vessels of the skin when the amount of gonadal tissue was diminished by three quarters.

DISCUSSION

Although hepatic tissue of rodents undoubtedly inactivates estrogens the evidence presented here indicates that the monkey's liver will not inactivate the estrogen produced by one-half of one ovary. The possibility that appreciable amounts of venous blood from the spleen bypassed the liver does not exist because the splenic vein in the monkey, as in the rodent, carries the major part, if not all, of the blood from the spleen (Hooker, Drill and Pfeiffer, 1947, Hartman and Strauss, 1933). Furthermore, it is not probable that lymphatics would transport effective amounts of hormone from the spleen or that the fenestrated sites of the grafts would transmit any of the hormone to the peritoneal fluids. Large pedunculated, estrogen-producing ovarian tumors growing from the spleens of mice, presenting a much larger peritoneal surface, do not often provide evidence of estrogen production unless the tissue acquires parietal adhesion (Li and Gardner, 1949).

Estrone and estradiol are destroyed in the monkey's body. Westerfeld and Doisy (1946) injected 3 to 6 mgm. of estradiol and estrone into intact, castrated or castrate-hysterectomized adult monkeys, and recovered only 1.5 to 5.3 per cent of the total amount in the urine

excreted. The possibility of excretion through the bile and intestinal tract was not investigated. As little as 2 per cent of injected estrone was recovered in the urine of human males and females (Pincus and Pearlman, 1942). The excretion of estrogens in the bile has been demonstrated in animals of some species (Cantarow *et al.*, 1942). If such occurs in man, and to a similar extent, the feces might contain appreciable amounts of the estrogen. Dingemanse and Lacqueur (1940) have found 60 to 70 per cent of the amount of estrogen excreted in the urine and in the feces of three healthy women. If in the monkey, the liver excreted estrogens, the effect upon the general sexual development of the hosts should have been much like that of castrated animals. Such was not true. The mechanism for the inactivation of estrogens in the monkey appears to be independent of the liver and quite unlike that of rodents which have been so extensively studied.

The possibility that transplanted ovaries produce an estrogenic compound differing from that which is produced by the normally located gonad, has been considered (Golden and Sevringhaus, 1938). If this consideration were correct, it would require that a compound resistant to hepatic enzymes and with similar end-organ reactions be produced. This assumption does not seem probable, especially since estrone is likewise not inactivated by the monkey's liver in amounts that could be detected (Drill and Pfeiffer, 1946). Similarly, it is improbable that the ovary draining its hormones more directly into the liver, would produce an inhibition of the estrogen-destroying system.

Dietary factors modify the estrogen-destroying capacity of the livers of rodents (Biskind, 1946; Drill and Pfeiffer, 1946). The general condition of the animals used in the present experiment was excellent and the responses of the uterus, vaginal mucosa and sex skin in all the animals with intrasplenic ovarian transplants were comparable to intact animals of similar ages.

Several investigators have observed the effects of partial or subtotal removal of the gonads upon accessory reproductive organs. Guinea pigs from which all of one ovary and an appreciable part of a second were removed often ceased to cycle and showed a marked uterine hypertrophy (Lipshütz, 1937, a & b). Although monkeys have not been studied over so long a period, they apparently ovulated when only 12.5 per cent of the ovarian tissue remained and they showed regular menstrual cycles as long as 8 months. The remaining fragments, however, showed considerable hypertrophy (van Wagenen and Morse, 1942). The reduction of ovarian tissue in monkeys without undue disturbance of the vascular connections, did not modify the pituitary-ovarian relationships unduly. Although evidence of formation of corpora lutea and of ovulation was not detected in the intrasplenic grafts at laparotomy, the rhythmic changes in the sex skin, vaginal desquamation and uterine bleeding all indicated normal function and implied a not greatly altered pituitary-ovary relationship.

The animals have been considered too valuable for subsequent study to allow removal of the transplants for detailed study at this time.

SUMMARY

Three immature females, two immature males and three mature female monkeys received, after castration, ovarian transplants so placed that in case of survival, the gonad would secrete into the portal circulation. The ovarian transplant in one immature animal was placed outside the immediate action of the liver, in the rectus abdominus muscle, as a control procedure.

All the female monkeys received homotransplants which survived and gave rich external evidence of hormonal action in supporting the menstrual cycles, cyclic vaginal desquamation and the sex skin color or swelling which is an external character so conveniently indicating hormonal states in this monkey.

The fate of the heterotransplants in the two immature males is not yet known for they are still immature. One of the two young males received ovarian tissue from a fetus and since this monkey is now (November, 1949) only two years and three months old, it cannot be expected to reach adolescence for another two years. If the transplanted tissue should become active, there are known responses in the male genitalia which will result from estrogenic stimulation.

CONCLUSION

The liver does not inactivate estrogens in the monkey (Macaca mulatta). In this particular, the primate is unlike the rodent whose physiological responses form the basis for so much of the present thinking.

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DIABETOGENIC EFFECT OF PURIFIED GROWTH HORMONE[1,2]

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THE PURPOSE of this investigation was to determine which components of the anterior pituitary gland possess diabetogenic activity. The production of diabetes in intact dogs by the administration of the extracts was taken as the index of this activity. The growth-promoting and adrenocorticotrophic activities of the various fractions were also determined. This study was aided greatly by the application of the methods of Wilhelmi, Fishman and Russell (1948) which made possible the preparation of a sufficient quantity of purified growth hormone. Purified adrenocorticotrophic hormone (ACTH) was prepared, and also three fractions, namely, APE and SC (which are diabetogenic) and APP (which produces fat mobilization).

METHODS

Diabetogenic activity

The production of definite hyperglycaemia, glycosuria and polyuria in intact dogs in 2 to 4 days following the first injection of the extract was the criterion of diabetogenic activity. In our experience, about 60 per cent of normal dogs are sensitive to the diabetogenic factors of the anterior pituitary. It is therefore necessary to select responsive animals by preliminary trials with APE, which is known to have diabetogenic activity. The dogs were maintained between tests on a diet of "Purina" dog chow, but for one day prior to, and throughout the test periods the daily diet consisted of 300 gm. ground lean meat, 100 gm. ground chow and 40 gm. sucrose given as two meals, at 10 A.M. and 5 P.M. Before each meal 1 cc. of blood was taken for the estimation of sugar (Somogyi, 1945). Subcutaneous injections of the test solutions were then given and the animals were fed. Usually injections were continued for 5 days. The urinary glucose was determined daily by the method of Benedict (1911) and Rothera's test was used for the detection of ketones. A minimum period of two weeks elapsed between the completion of one test and the beginning of the next. In general, the injections were well tolerated, but if a severe diabetes developed the dogs became depressed and irritable, and their appetites decreased. The responsive dogs, P, F and W,

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which have been in the laboratory for 4, 3 and 1 years, have been used 20, 13 and 9 times respectively for testing purposes, and remain in good condition.

These tests give information concerning the comparative activities of various preparations, but not enough data are available for a quantitative assay. It would appear that neither the peak of the increase in blood sugar nor the total weight of sugar excreted, taken singly, gives an entirely satisfactory estimate of diabetogenic activity. In the absence of sufficient dose-response data, only the sugar excretion in grams per 10 kg. dog for the 5-day test period will be compared.

Growth activity

Using the method of Marx, Simpson and Evans (1942), female rats of the Wistar strain from 70 to 100 gms. body weight were hypophysectomized and after a recovery period of 12 to 15 days, during which the body weight became more or less constant, were injected intraperitoneally each morning with 1 cc. of the test solution until 10 injections had been given. The body weights were recorded daily and the gain in weight in the ten-day period following the first injection was determined. The percentage composition of the diet was:—whole peanut meal 35; oatmeal 35; skim milk powder 15; "Cerogras" (dried young grasses) 5; dried brewer's yeast 2; salt mixture (according to Beveridge and Lucas, 1945) 2; corn oil 3; wheat germ oil 2; cod liver oil 0.1 and choline chloride 0.05.

Adrenocorticotrophic activity

The adrenocorticotrophic activity was assayed by a slight modification of the "difference" method of Sayers, Sayers and Woodbury (1948) as shown in Table 3. Immediately after excision the adrenal glands were weighed and placed in freshly prepared 3% metaphosphoric acid at 2°C. The ascorbic acid contents were determined by the method of Bessey (1938). The reagents were prepared with glass-distilled water which had previously been boiled to free it substantially of dissolved oxygen.

Preparation of extracts

The preparation of the growth hormone fraction was carried out in a cold room at 2° to 5° C. The ethanol used for the extraction was purified by distillation with sodium hydroxide and again with calcium oxide. From 486 grams of freshly frozen anterior pituitary glands of cattle, 32.8 gm. of Fraction A were obtained by the method of Wilhelmi, Fishman and Russell (1948). Fractions B and C together weighed 4.74 gm. but were not used at this time. The further purification of Fraction A yielded the final, white, irregularly crystalline growth hormone precipitate (W.4) which, when lyophilized, weighed 3.16 gm. This product was further purified. It was dissolved in 1% potassium chloride to give, approximately, a 0.8% solution at pH 11. The solution was adjusted to pH 8.7 and was dialysed against running distilled water at pH 6.8 in the cold for 16 hours. The precipitate was redissolved and redialysed, and the resulting precipitate, when lyophilized, weighed 1.60 gm. This product was termed the growth hormone W.4.P.

The ACTH, S.10, was prepared by the method of Fishman (1947) from hog pituitary glands. Comparison with the ACTH, Lot 41-L of the Armour

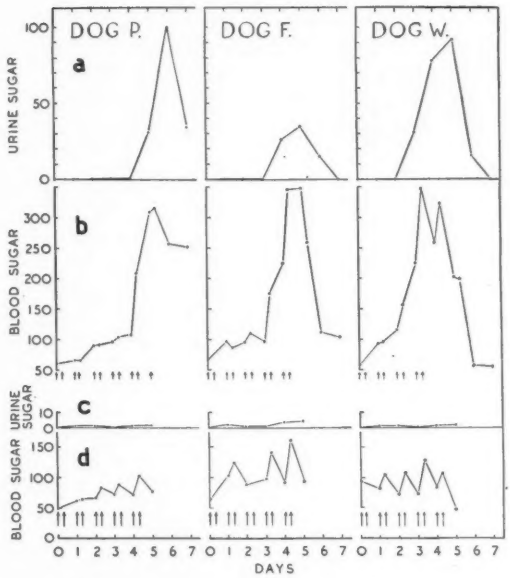

FIG. 1. a to d:—tests of diabetogenic activity in fed, adult, male dogs. Each arrow in a and b represents the subcutaneous injection of 1.75 mg. growth hormone W.4.P. per kg. and, in c and d 3.75 mg. ACTH S.10 per kg. The blood sugar values are expressed as mg. per cent and the urine sugars as gm. per dog per day.

Laboratories[3] (Table 3) indicated that the adrenocorticotrophic activity of ACTH, S.10 was satisfactory. The oxytocic activity of the latter was definitely less than 0.1 unit per mg.

The APE, D.15, was prepared in essentially the same way as the globulin extract of Campbell and Keenan (1940). Details concerning this will be given along with the preparation of the SC (semi-crystalline) fraction in a later publication. The APP, described by Best and Campbell (1936), was prepared at 2°C. When solutions were prepared for injection from dried fractions which were not completely soluble at pH 7.5, i.e. APE, SC and APP fractions, the amount of protein in solution was calculated from the total nitrogen content (determined according to the method described by Umbreit, 1945).

RESULTS

Diabetogenic activity

It is apparent that the growth hormone gave rise to a pronounced diabetes in the 3 test dogs (Figure 1; a,b). The blood sugar rose gradually in the first 2 to 4 days of treatment, then rapidly to above 300 mg. per cent, this increase being well sustained. The peak values

[3] The ACTH, Lot 41-L, kindly donated by the Armour Laboratories, was said to contain 68% of the adrenocorticotrophic activity of ACTH, La-1-A, which is referred to in the article of Sayers, Sayers and Woodbury (1948).

for sugar excretion were 31, 34 and 92 grams per dog per day in a 5 day period. Ketones were present in the urine towards the end of the experiment. The amounts of growth hormone required to elicit these responses (3.5 mg. per kg. per day) were relatively small. The results of these tests are summarized with the data on the other preparations in Table 1.

TABLE 1. TESTS OF DIABETOGENIC ACTIVITY IN INTACT MALE DOGS

Preparation injected	Dose per kg. per day mg.	Dog	Initial body weight kg.	Initial blood sugar mg. %	Peak values in 5 days			Total urine sugar per 10 kg. in 5 days gm.
					Blood sugar mg. %	Urine sugar per 10 kg. per day gm.	Urine volume per 10 kg. per day cc.	
Growth	3.5	P.	16.0	60	310	19	332	19
Hormone	3.5	F.	9.5	68	344	36	1610	63
W.4.P.	3.5	W.	11.1	62	348	83	1660	180
APE	3.5	P.	16.0	77	71	0.8	150	3
D.15	3.5	F.	9.8	94	132	1.7	300	4
	3.5	W.	10.0	83	225	15.7	370	17
APE	7.5	P.	14.8	60	306	47	1490	78
D.15	7.5	F.	10.0	87	308	39	1500	64
	7.5	W.	11.1	56	278	76	1036	133
SC*	7.5	P.	13.2	60	219	81	1590	122
	7.5	F.	9.3	72	285	40	1720	87
	7.5	W.	10.5	65	261	83	2120	131
ACTH	7.5	P.	13.7	49	102	1.2	292	2.5
S.10	7.5	F.	9.9	65	164	3.1	718	8.2
	7.5	W.	11.4	92	129	1.4	561	3.2
APP	7.5	P.	15.3	78	76	0.5	131	1.4
	7.5	F.	9.4	68	87	0.5	245	1.1
	7.5	W.	9.8	46	84	1.4	868	2.0

* These values are for a 4-day test period only.

The administration of the APE and SC fractions to the test dogs consistently elicited diabetic responses which were of the same character as those produced by the growth hormone, but weaker. Daily doses of 3.5 mg. APE gave negative or slightly positive responses, while 7.5 mg. gave strong responses which were estimated to be about equivalent to those produced by 3.5 mg. of the growth hormone W.4.P. The SC fraction appeared to be about as active, or more active, than the APE fraction. From this it is estimated that the APE and SC fractions were roughly half as active as the growth preparation.

The ACTH produced some elevation of blood sugar and a slight glycosuria. These responses were more rapid but more fleeting, giving the blood sugar curves a "saw-toothed" appearance in contrast to the sustained, high curves obtained by treatment with the growth hormone (Figure 1; c,d). The diabetic states produced by the growth

hormone, the APE and the SC fractions appear, therefore, to be different both in intensity and in time relations from that produced by the ACTH. Treatment with APP did not elicit definite signs of diabetes.

Growth-promoting activity

The growth hormone, W.4.P. gave growth responses (Table 2) in good agreement with those which Li, Evans and Simpson (1945) obtained with their pure growth hormone (Figure 2). Since the relationship of the response to the log. of the dose is apparently linear, it may be expressed as gain = a log. dose + b, where gain is the weight increase (gm. per rat in 10 days) and dose is the amount of material

TABLE 2. IN THESE ASSAYS OF GROWTH-PROMOTING ACTIVITY, FEMALE HYPOPHYSECTOMIZED RATS WEIGHING 70–100 GM. WERE GIVEN DAILY INTRAPERITONEAL INJECTIONS OF THE TEST SUBSTANCE IN 1 ML. SOLUTION FOR 10 DAYS

Preparation	Dose per rat per day μg.	No. of rats	Average body weight gain in 10 days per rat gm.	Standard* error
None (controls)	0	8	−0.38	±1.44
Growth hormone, W.4.P.	10	8	11.5	±1.32
	25	11	16.2	±1.40
	100	10	21.2	±1.45
APE, D.15.	20	4	12.5	
	50	5	14.6	
	200	4	8.8	
SC	20	5	8.8	
	50	4	15.4	
APP	50	4	2.5	
	100	3	8.0	
	250	4	10.2	

* Standard error $= \sigma/\sqrt{n}$, where σ = the standard deviation, n = the number of observations.

injected (μg. per rat per day). The constants, calculated by the method of least squares, from the results of the present study, from those of Li, Evans and Simpson (adjusted to cover a 10-day period) and from both combined are, a = 10, 11 and 10.8 respectively; b = 1.6, −0.3 and 0.5 respectively. Since the intercept (b) on the "gain" axis is negligible, the equation becomes, approximately:— gain = 11 log. dose.

A comparison of the pituitary preparations (Table 4) shows that the diabetogenic effect corresponded roughly with the growth-promoting activity. The ACTH, however, had a slight diabetic effect but had no growth activity; in fact, it caused the test animals to lose weight.

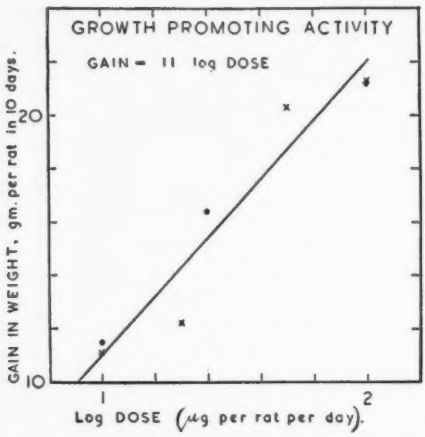

FIG. 2. Growth produced in young, female, hypophysectomized rats by pure growth hormone according to Li, Evans and Simpson (1945) —×— (4 points); and by the growth hormone W.4.P. used in the present study —•— (3 points). The duration of the test, counting from the first injection, was 10 days.

Adrenocorticotrophic activity

Assays showed that the ACTH, S.10, had much greater adrenocorticotrophic activity than any other preparation tested (Table 3). When this preparation was assigned the value of 100 for this activity, the growth hormone W.4.P in comparison showed about 1.6% (Table 4). Apparently this contamination was not enough to interfere seriously with the growth-promoting and diabetogenic effects of W.4.P.

TABLE 3. TESTS OF ADRENOCORTICOTROPHIC ACTIVITY

Male rats of 122 to 168 gm. body weight were hypophysectomized. Twenty-two to 26 hours later the left adrenal was removed under amytal anaesthesia. The body weight fell by about 14 gm. in this interval. The substance to be tested, made up in 1 cc. saline solution, was injected into the femoral vein. After 1 hour the other adrenal gland was removed and the ascorbic acid concentration in each gland was determined.

Preparation	Dose per 100 gm. body weight	No. of rats	Ascorbic acid concentration		Standard error of the depletion
			Left adrenal mg. %	Depletion mg. %	
ACTH, S.10	2	6	432	143	±15.0
	0.5	6	417	98	±13.6
Growth Hormone, W.4.P.	100	6	410	149	±16.2
	20	5	398	67	±18.2
	12	5	420	59	±19.2
APP	18.9	7	437	68	± 8.93
	6.3	7	411	18	± 8.40
ACTH, 41-L	4	6	417	102	± 5.6
	1	7	440	43	± 6.7

TABLE 4. SUMMARY OF THE COMPARATIVE ACTIVITIES OF THE FIVE PREPARATIONS

The growth hormone W.4.P. was assigned the value of 100 for diabetogenic activity and also for growth-promoting activity. The ACTH, S.10, was assigned the value of 100 for adrenocorticotrophic activity.

Preparation	Diabetogenic activity	Growth activity	Adrenocorticotrophic activity
Growth hormone, W.4.P.	100	100	Slight (1.6)
APE, D.15	Estimated approx. 50	55	Slight (0.7)
SC	Estimated approx. 50	43	Slight (0.5)
APP	0?	4	Slight (1.7)
ACTH, S.10	Slight	(Weight lost)	100

The APP had 1.7% adrenocorticotrophic activity and the APE and SC fractions even less. It is clear that the diabetic responses to the various preparations cannot be attributed to their contents of ACTH.

Discussion

As described by Houssay (1942) and others, the administration of the diabetogenic factors of anterior pituitary extracts to dogs results, after a latent period of 2 to 4 days, in a marked, sustained rise of blood sugar, with polyuria, glycosuria and ketonuria. This response was elicited by the growth hormone, APE and SC fractions studied. The crystalline growth hormone preparation, which was highly purified, had the highest diabetogenic activity of any of the preparations which have been tested in this laboratory. A comparison of the growth-promoting with the diabetogenic activities of the preparations suggests strongly that, in the dog, these activities run roughly parallel.

In addition to the results reported herein, some data on the amount of diabetogenic activity in bovine anterior pituitary glands are available. Experiments performed previously by one of us (J.C.) showed that the daily intraperitoneal injection of a crude, saline extract of anterior pituitary glands elicited, in 7 intact dogs, typical diabetic responses when the average dose was equivalent to 1.5 gm. (range 1.2 to 1.7 gm.) of fresh glands per kg. per day. This agrees with the results of Houssay, Biasotti, Benedetto and Rietti (1934) who found that 1.4 gm. of glands were effective. The relatively purified globulin fraction, APE, produced strong diabetic responses in a daily dose of 7.5 mg. per kg. This dose was derived from an equivalent of about 1.5 gm. of anterior pituitary glands, since the yield of APE from the fresh glands was 0.5%. This comparison is rough, but it suffices to indicate that equal weights of fresh anterior pituitary

glands are required to produce effective diabetogenic doses of either crude saline extract or of globulin fraction, APE. If it be assumed that most of the diabetogenic activity of the anterior pituitary glands is present in the crude saline extract, then most of this activity is also found in the relatively purified APE fraction. This conclusion is supported by fractionation studies which showed that in the preparation of APE there were only small losses of diabetogenic activity in the other fractions, and that most of the active material was extracted. In the present studies it was found that the diabetogenic activity of the APE corresponded roughly with its growth activity. Thus it appears that the major part of the total diabetogenic activity of bovine anterior pituitary glands can be attributed to the growth hormone which they contain. It seems unlikely that another substance more active than the growth hormone in producing diabetes in dogs will be found in these glands in appreciable amounts. The daily effective diabetogenic dose of growth hormone W.4.P per kg. dog was obtained from about 1 gm. of bovine anterior pituitary glands. This equivalent of glands is less than those cited above for crude saline extract and for the APE fraction.

The signs of diabetes elicited by the ACTH were not very pronounced and the response was also qualitatively different from that obtained with growth hormone, APE and SC fractions in that the rise in blood sugar appeared earlier, yet faded relatively quickly. Since the "diabetes" produced by the ACTH differs so greatly from that produced by the crude extracts, growth hormone and certain other fractions of the pituitary gland, it would perhaps be better not to refer to the response to ACTH in dogs as "diabetogenic" until more is known of the nature of the changes induced.

It is possible that the diabetic effect of purified growth hormone might be enhanced or inhibited by other pituitary factors. Little is known as yet of the rôle of the anterior pituitary gland in the etiology of human diabetes, and the possible effects of more than one pituitary hormone must be considered in this regard.

It appears that Evans, Meyer, Simpson and Reichert (1932), who produced diabetes in dogs by the prolonged administration of a crude growth preparation, were the first to indicate a relation between growth hormone and diabetogenicity. Cotes, Reid and Young (1949) concluded recently that pure growth hormone is diabetogenic in the intact, adult cat. Current findings do not establish that the growth and diabetogenic activities are indivisible. The results of the present study, which show that highly purified growth hormone is diabetogenic in the dog, provide further evidence which points in this direction.

SUMMARY

In intact dogs, highly purified, crystalline growth hormone in a daily dose of 3.5 mg. per kg., after an interval of 2 to 4 days, elicits

marked diabetic responses, with hyperglycaemia, glycosuria, polyuria and ketonuria.

Evidence is presented which indicates that, for the dog, the growth hormone is the important diabetogenic substance of bovine anterior pituitary glands, both with respect to activity and to the total amount present in the gland.

The ACTH elicits signs of diabetes (hyperglycaemia and glycosuria) which are much less intense than those produced by the growth hormone. The "diabetes" produced by ACTH in dogs differs in character and in severity from that produced by the growth hormone.

ACKNOWLEDGMENT

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"SCOTTIE"—TWELVE YEARS DIABETIC

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In 1937 two pancreas operations were performed upon a mature-appearing male dog of predominantly Scotch terrier ancestry. The age was obviously above one year and perhaps above two years. The method of partial pancreatectomy described long ago (Allen, 1913) involves trimming a piece of the removed tissue to duplicate the remnant left around the main duct, as closely as can be judged by both sight and feeling. Anyone who practices this method on recently dead animals, or as a stage in total pancreatectomy, weighing the estimated remnant and the total removed, can establish the accuracy to within a fraction of a gram. The remnant in this animal at the first operation was thus estimated at 3.4 gm., or one-ninth of the pancreas. Permanent diabetes did not occur until a second operation removed 0.4 gm. of additional tissue. The diabetes then was controllable by brief fasting, but after some experiments involving glycosuria the dog could no longer be starved sugar-free. Anatomically, this condition is regularly accompanied by loss of all beta cells through hydropic degeneration, leaving only small clumps of strongly granulated alpha cells amid the acinar tissue.

Chiefly by his attractive personality, this dog gained the status of a house pet. On protein diet the feces were bulkier than normal, and sometimes soft. Milk or any form of fat caused diarrhea. Excellent health was maintained on a diet of 300 to 400 gm. of lean horsemeat, chopped and autoclaved, mixed with a little talcum powder, and divided into three or four feedings daily. Only trivial extras of table meat or vegetables were occasionally obtained by begging. One Uneeda biscuit was given every night partly because of the dog's strong desire for it and partly for protection against nocturnal insulin reactions. On this regime the weight was kept at 20 pounds, with variations of no more than one pound up or down, except for one transitory rise to 22 pounds and one fall to 18 pounds in a diarrheal attack.

Aside from a few experiments involving increased dosage, the insulin varied between 30 and 40 units, divided into three injections

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daily, the dog coming for his injections eagerly because of the accompanying feeding. Only plain or crystalline insulin could be used, the sugar levels being too unstable for control by protamine or globin insulin. The sugar was thus regulated as closely as possible, though it is not possible to keep a partially depancreatized dog under as thorough control as a human diabetic. Glycosuria or hypoglycemia occurs without warning and without causation by any known change of diet, insulin or exercise. The mentioned changes in diet or insulin represented adjustments to check these conditions. Slight insulin reactions, averaging two or three weeks apart, were recognized by depressed behavior and cleared up by feeding a trifle of carbohydrate. About three or four times each year, most often by night, there was severe hypoglycemia with unconsciousness and convulsions, cured by subcutaneous injection of 2 grams of glucose.

In 1949 the dog was at least 14 years old. There were signs of age in the spontaneous loss of two teeth, discoloration of others and some recession of gums, graying of hair at muzzle and rump, and slight impairment of vision due to beginning cataract. The hair was as abundant and glossy as ever, and the striking difference from most old dogs was the retained liveliness, with jumping as high and enthusiastic as ever.

Three months of declining appetite and strength were mistakenly attributed to senility. On August 4, the dog fell when attempting to walk and died within 3 hours, the decline being unaltered by a subcutaneous glucose-saline injection.

In the immediate autopsy, the peritoneum contained a large quantity of brown turbid purulent fluid, evidently the result of a pin-head perforation of an abscess about the size of a full stomach, for which it was mistaken at first glance. It was irregularly thick-walled and completely isolated in the omentum, without connection or adhesion to other organs. The pancreas remnant weighed 3.2 gm. and was composed of normally soft tissue, practically free from adhesions because of the usual covering with peritoneum at operation. The close matching with the original estimated weight indicated absence of important atrophy or hypertrophy. The skull was not opened. Otherwise a thorough search revealed no gross abnormality anywhere. The viscera and vessels appeared as fresh and perfect as those of a young dog.

A little urine in the bladder gave a moderate sugar reaction, due to the injection, but there was no acetone or albumin. Analysis of heart blood showed sugar 189, urea 28, creatinine 1.1 mg. per cent; qualitative acetoacetic test negative. Cholesterol analysis was not done, but the absence of lipoid deposits in organs was evidence against an excess. Tissue specimens were taken in 4 per cent formaldehyde, sectioned and stained in ordinary manner.

The pancreas was typical with its wide expanses of acinar tissue

without recognizable islands. No special staining was done. One section of skeletal muscle showed an area of diffuse necrosis with purulent infiltration. Heart muscle, lungs, liver, spleen, adrenals and kidney were normal, also the retina. Large and small blood vessels were strictly normal, without any sign of atheroma. The diagnosis was death from acute infection.

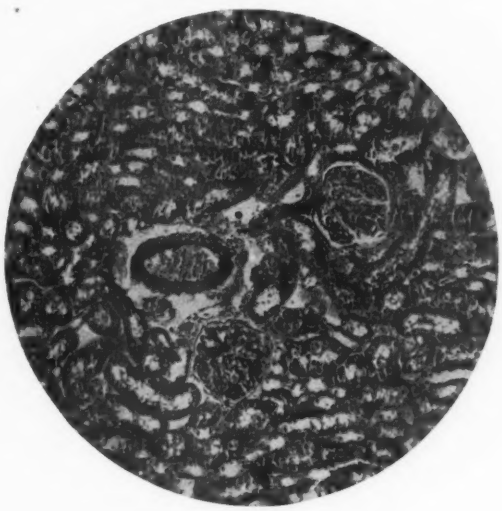
COMMENTS

The omental abscess had structural signs of fairly recent origin. It was only possible to surmise a causation from perforation of the bowel by some object such as a sharp spicule of bone, though no foreign body was found. The abscess formation and final perforation were the obvious cause of the failing health and acute death.

The behavior of the animal up to this time, together with the autopsy findings, gave a prospect for a considerably longer life except for this accident. Most dogs of this small type are obese, sluggish and more or less decrepit at the age of 14 years. This dog was kept moderately thin and always hungry. The continued liveliness and ebullience suggested an actual prolongation of life by undernutrition, such as several writers have demonstrated in rats and mice.

In the past there have been speculations that arteriosclerosis or nephritis found in carnivorous animals is due to their protein diet. This example shows that a nearly unmixed meat diet need not cause vascular or organic disorders in a dog.

Totally depancreatized dogs are known to die with fatty livers after a few months of treatment only with insulin. The presence of a small remnant of pancreas, sometimes a twelfth of the gland or less, prevents this disorder. The present experiment shows that a small pancreas remnant enables a dog to keep a normal liver throughout twelve years of diabetes, without any dietary supplement.

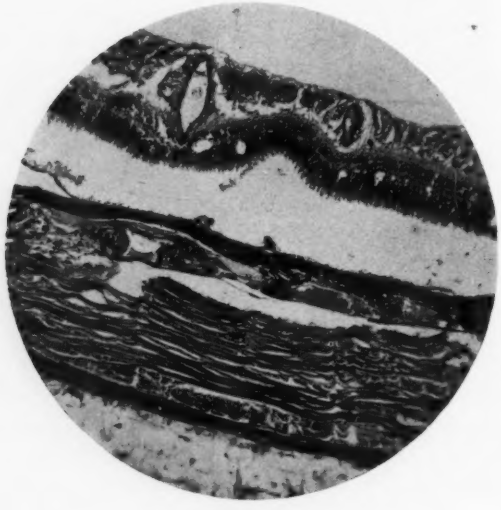


Kidney

Previous experience has shown that without insulin administration the partially depancreatized dog, even after all beta cells have disappeared, has no such lack of resistance to infection as the totally depancreatized dog. Abdominal operations can be performed practically as safely as in normal dogs, even though carbohydrate feeding may produce greater glycosuria and hyperglycemia than in the totally depancreatized dog. This agrees with the understanding that the mere surplus of glucose is not the cause of liability to infection in human patients. It also shows definitely that a small mass of acinar, alpha or other pancreatic cells has some kind of powerful influence upon resistance in dogs.

The importance of the pancreas is known to vary widely in different species. Totally depancreatized dogs and cats shown not only

extreme diabetes, cachexia and fatty liver but also serious impairment of digestion. The present dog developed diarrhea from any kind of fat, even cream, and therefore required a diet without gross fat content. Asphyxial inflammation of the pancreas remnant (Allen, 1922) can selectively destroy islands so as to bring on diabetes with larger remnants and consequently with better digestive function. The depancreatized human subject is more like some other animal species, in developing much milder metabolic or digestive disturbance. A woman now under the care of one of us, following a successful com-

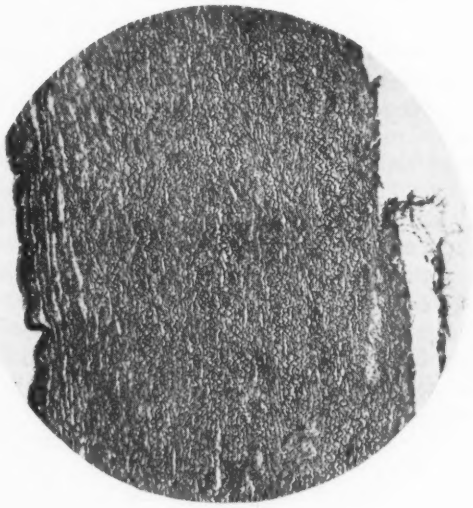


Retina

plete pancreatectomy for cancer, conforms to the descriptions in the literature in having a very moderate diabetes controlled with 16 to 26 units of daily insulin, adequate digestion without artificial aid, and no apparent tendency to infection, fatty liver or cachexia.

It would be instructive to learn whether such a patient can be starved sugar-free. The totally depancreatized dog cannot be sugar-free except in a cachectic stage which is incompatible with life, and the partially depancreatized dog with less cachexia is even more stubbornly glycosuric. Amid the confused reports concerning pancreatic islands in diabetic autopsies, there was one clear impression from a study before the time of insulin (Allen, 1922); namely, if abundant islands are found, death is unnecessary as far as diabetes is concerned. That is, such islands always have enough function to permit keeping the patient alive on reduced diet. Moreover, it appeared as a definite fact that a patient with spontaneous diabetes

cannot live without pancreatic islands. There were a few such patients, especially children, who could not be sugar-free even with the most extreme diet reduction and emaciation, furnishing rare examples of absolute choice between death from diabetes and death from starvation. All autopsies obtained on such cases furnished an explanation in the form of absence of islands, or only a few small remains with shrunken atrophic cells that were obviously functionless (Allen 1922; Allen and Sherrill, 1922). In far more cases the choice was hard but not absolute. Children and adults were kept alive with diets and



Aorta Wall

body weights which today seem incredibly low, and later for good nutrition they required something like 60 to 90 units of insulin daily, in other words two or three times as much as a totally depancreatized patient. Stringent fasting of a depancreatized patient might establish one of two facts, either of which would be remarkable. (a) A human being can live and be sugar-free on some reduced level of nutrition, if free from the special added influence which is responsible for the excessive insulin requirement in severe spontaneous diabetes. (b) Or, the depancreatized human subject may, like the dog, be unable to live at any nutritive level; in other words, he has a greater absolute severity of diabetes than anybody who still possesses some islands, as revealed by this test which is a truer measure of severity than the insulin dosage required to maintain high nutrition.

It should scarcely be necessary to add that fasting never injures the islands, nor does high carbohydrate feeding strengthen them.

This false supposition still crops up occasionally, notwithstanding the preexisting proofs (Allen, 1913) that: (a) the islands remain anatomically intact and glucose is well assimilated even in the most extreme starvation when the acini have lost nearly all their zymogen granules; (b) a "border-line" animal is one which can be made diabetic by carbohydrate feeding, either immediately after operation or later. If the islands were impaired by fasting, a starvation period should increase this susceptibility. Actually, it conduces to recovery, so that sudden forcing of carbohydrate results only in brief "hunger glycosuria" and refractoriness to persistent diabetes. The misnamed "hunger diabetes" and the more recently demonstrated reduction of insulin content of the pancreas with fasting represent merely a temporary adaptation to reduced demand. The falsity of the supposition that either fasting or fat diet lowers the true assimilative capacity or the island function is best proved by attempts to produce diabetes in a partially depancreatized dog.

The keeping of this particular dog in a household was the only way for fairly strict control of the sugar, because of the many occasions when unexpected hypoglycemia would have been fatal under the less constant watch in a laboratory. On the other hand, the lack of laboratory facilities precluded a number of studies which could have been interesting. One of these might have compared carbohydrate assimilation in human and canine diabetes. The influence of total calories and body weight was first shown in dogs (Allen, 1914; 1920) and confirmed in human patients (Allen, 1915, 1920; Allen, Stillman and Fitz, 1919) then after the advent of insulin the requirement created by different classes of foods was measured in units of insulin dosage (Allen and Sherrill, 1922; 1923). It was thus found that isocaloric substitutions of carbohydrate and fat make comparatively slight change in the insulin requirement. On the other hand, reduction of obesity improves assimilation far more than mere carbohydrate restriction; and conversely, increases of body weight with fat or other calories multiply the required insulin dosage altogether out of proportion to any effect of high carbohydrate with low calories. Besides the numerous clinical experiments, there was one instance of practical use of a ration of 250 gm. of carbohydrate in a case of diabetes with nephritis (Allen and Sherrill, 1922). In general the very high carbohydrate diet was rejected, not because of an excessive demand for insulin but because of the more difficultly controllable fluctuations of blood sugar (Allen and Sherrill 1922, 1923; Allen, 1928), and this objection still remains valid. Some later writers introduced one-sided diets as if they were a new discovery, but demonstrations of thorough control and ultimate safety with this plan appear to be still lacking. .By contrast, the partially depancreatized dog seems disproportionately sensitive to carbohydrate. Even without means for accurate studies, carbohydrate obviously caused greater glycosuria in the pres-

ent dog than its protein equivalent. Particularly, attempts at maintenance on an isocaloric ration of cereal or dog-meal were impossible because of the high and fluctuating sugar and the excessive insulin requirement. If this observation can be more exactly confirmed, it may signify a difference in the metabolism of the diabetic dog and man.

On the other hand, increasing experience has confirmed the general close similarity between clinical diabetes and that of the partially depancreatized dog. Studies on such dogs were the first basis for: (a) proposing normal blood sugar as the standard of diabetic control; (b) suggesting hydropic degeneration of islands as the usual or only cause of decline of tolerance; (c) contradicting the previously universal belief in "spontaneous" downward progress and attributing such progressiveness to overtaxed function. This comparison between man and dog and clinical applications of the concepts based on it were stressed repeatedly in publications before and after the discovery of insulin (Allen, 1915, 1920; Allen, and Sherrill, 1922, 1923). For example: "So far as known, youthful patients with continuous hyperglycemia have invariably progressed downward." "If there is any hope of preventing downward progress in typical youthful cases of diabetes, it can be found only in those whose assimilative function is spared to the extent of maintaining a constantly normal blood sugar." "The observations in this series have created the impression that if diabetic patients could be shut up in cages and fed strictly according to theoretical indications, downward progress would be as little evident in typical human cases as in experimental dogs." Though it is still not certain to what extent the arteriosclerosis and other "comcations" of diabetes can be duplicated in dogs, clinical experience and general theory caused early emphasis upon the principle of normal blood sugar for the prevention of all "complications," as illustrated in the above-cited references. For example (Allen and Sherrill, 1922): "The writers have previously placed emphasis upon the fact that not a single diabetic complication has ever occurred in any of these cases under thorough dietetic control." Other quotations are given in an ensuing publication.

If the method of experimental reproduction of arterial and other "complications" is feasible, it can offer more precise proof than clinical observations. Being the only fragment that could be saved, the present experience of keeping a severely diabetic animal for twelve years without organic or vascular pathology stands as an isolated fact, for lack of controls in various directions. The part played by control of the sugar could only be proved by comparative experiments with different diets and different controls. Granting control to be a factor, there may be an argument that the approximate control in this instance was as good as thorough control. Neither the high protein nor the near-absence of carbohydrate caused arteriosclerosis, but some

persons may see the lack of fat as the saving feature. Dogs with large pancreas remnants, or perhaps with alloxan diabetes, would be needed for tests with fat diet. If experiments along these lines had been possible, the problem of diabetic arteriosclerosis might have been considerably clarified.

CONCLUSIONS

A dog with severe diabetes after partial pancreatectomy was able to live for twelve years under control of the sugar with insulin, without organic or vascular pathology. As far as known, this is the longest recorded survival with any form of severe experimental diabetes.

The retention of unusual liveliness, together with the normal autopsy findings, indicated that life could have been considerably longer except for an accidental termination. The controlled diabetes evidently did not reduce longevity. The continuous limitation of calories and body weight is suggested as possibly prolonging both life and vigor, in accord with demonstrations in other species.

Some interrelations of human and canine diabetes are discussed. Differences of species must be considered in interpreting experiments. The present observation can stand only as an isolated fact, until suitable control experiments along the lines indicated may elucidate the cause of diabetic arteriosclerosis.

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THE EFFECTS OF TESTOSTERONE ON THE CONNECTIVE TISSUE OF THE COMB OF THE COCKEREL[1]

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THE GROWTH of the cock's comb has long been known to be dependent upon stimulation by androgens (Champy and Kritch, 1925; Hardesty, 1931). By virtue of this reaction the combs of the capon and chick have been used extensively for the bioassay of male hormones. Remarkably, however, there has been little attempt to analyze the structural changes in this response.

In 1925–26, Champy and Kritch published the first histological descriptions of the comb of the chicken. They identified a "muco-elastic" layer in this organ, consisting of loose connective tissue in which there were abundant elastic fibers and many large vacuoles filled with mucoid material. They claimed that complete disappearance of this layer occurred after castration, and that it reappeared after intraperitoneal transplantation of testicular tissue.

Hardesty (1931) published a more detailed histologic description, in which she subdivided the comb into several anatomic layers. We have confirmed her findings and have adhered to her terminology throughout. She considered the adult cock's comb to consist essentially of epidermis and dermis. The latter in turn was subdivided into three layers: central, intermediate, and peripheral. (1) The central zone is a core-like structure of parallel vertical collagen bundles, containing adipose tissue, large blood vessels, and nerves. (2) The intermediate zone, corresponding to Champy's muco-elastic layer, is a loose network of collagen fibers and fibroblasts, with large deposits of intercellular mucoid material which stains metachromatically with certain basic dyes. Contrary to Champy, Hardesty points out that this layer does not disappear entirely after castration, but merely becomes smaller because of the loss of the intercellular ground substance. (3) The peripheral zone or layer is a narrow band of somewhat denser fibrous tissue with an angiomatoid network of distended capillaries underlying the epidermis.

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[2] Charles Klingenstein Fellow.

The cock's comb, then, is a structure providing an abundance of connective tissue ground substance which is profoundly influenced by androgens. We have therefore used this organ as a means of studying the effect of testosterone on ground substance.

METHODS

The animals used were single-combed White Leghorn cockerels shipped directly from a commercial hatchery within twenty-four hours of hatching. They were kept in a battery brooder with thermostatic controls and were fed a stock chick starter mash (Quaker Oats Company). For each experiment the animals were divided into control and testosterone-treated groups of approximately equal size. At the conclusion of the experiments the chicks were killed with ether. The combs were dissected according to the technique described by Frank, *et al.* (1942), weighed on a Roller-Smith torsion balance, and examined histologically. Formalin, Zenker, basic lead acetate, Carnoy, acetone, and alcohol fixation were tried at the outset, but subsequently 20% formalin and 95–100% alcohol were used routinely. It was found that since much of the metachromatic material is slowly soluble in water, prolonged fixation in aqueous solutions, or washing of the tissues, resulted in varying degrees of loss of metachromatic substances. The tissues were embedded in paraffin and cut at 5 micra. The stains used were hematoxylin and eosin, toluidine blue, methyl green-pyronine, Mallory's trichrome connective tissue stain, vanGieson, periodic acid-fuchsin, and Gomori's alkaline phosphatase technique.

Sections of combs from the experimental animals, as well as sections of adult cock's combs, were subjected to digestion with bull testis and streptococcal hyaluronidase[3] adjusted to concentrations of 40–50 turbidity reducing units per cubic centimeter for 18 hours at room temperature. These were stained with toluidine blue and compared to untreated sections stained in the same manner. Similarly fixed sections of human umbilical cord were used as controls and were subjected to enzyme digestion and staining simultaneously with the comb sections.

RESULTS

In each of the experiments, the comb weights and the comb-body weight ratio (a commonly used correction factor) of the treated chicks were significantly greater than the control groups. The increase in comb size also varied directly with the dose of the testosterone administered (see Table 1).

In Experiments 1, 2, and 3, groups of three day old male chicks were treated daily for eleven days with 10γ, 50γ, and 100γ of free testosterone[4] respectively. On macroscopic examination the combs of the treated chicks were much larger than those of the control groups and revealed microscopically much greater differentiation into the adult structure described above.

The control combs consisted of a poorly defined, narrow central

[3] Streptococcal and bull testis hyaluronidase obtained through the courtesy of Dr. J. Seifter, Wyeth Research Laboratories, Philadelphia.

[4] Testosterone supplied by Ciba Pharmaceutical Products, Inc.

zone, containing little or no fat. The intermediate layer was made up of closely packed bundles of collagen fibers. The fibroblasts were small young cells with scanty, slightly basophilic cytoplasm, which stained only moderately well with pyronine. No metachromatic material was evident in sections stained with toluidine blue. The peripheral zone was not yet present.

In the combs from the treated animals, the central zone was more sharply defined and contained a considerable number of fat cells. At the outer portions of this layer many young, incompletely differen-

TABLE 1

Experiment	Number of animals		Age when killed	Dose (γ)		Body weight (grams)		Comb weight (mg.)		Comb weight / Body weight ×100	
	C	T		C	T	Control	Treated	Control	Treated	Control	Treated
1	18	12	14	0	10	135.4± 3	138± 4	176± 22	265± 31	129± 15	189± 18
2	11	14	14	0	50	127 ± 7	128± 6	149± 21	315± 29	112± 13	245± 19
3	22	17	14	0	100	117 ± 3	112± 3	101± 8	323± 22	86± 6	289± 18
4	3	3	18	0	100	216 ±18	228± 4	901±260	2016±225	412± 96	883± 83
5-a	8		3	0		33 ± 1		12±0.7		36± 2	
b	4	4	7	0	100	60 ± 2	62± 2	20± 2	33± 3	33± 3	54± 2
c	4	4	12	0	100	89 ± 4	89± 5	39± 4	119± 16	44± 4	132± 10
d	4	4	16	0	100	122 ± 9	125± 6	130± 18	421± 17	106± 9	335± 5
e	4	4	20	0	100	162 ±15	170±10	408± 53	792± 54	252± 23	463±0.5
f	4	4	24	0	100	216 ±12	222±10	818±157	2080±389	382±125	926±156

tiated fat cells of the "mulberry" type were seen. The central and intermediate layers were clearly separated from each other by a vertical band of dense connective tissue. The most striking change took place in the intermediate layer, which became much wider and occupied a relatively greater proportion of the total comb width. The structure was much looser and less compact; the collagen bundles were widely separated by large amounts of amorphous interfibrillar and intercellular material, which stained metachromatically with toluidine blue. This material was not stained by the periodic acid-fuchsin method. The fibroblasts were more numerous than in the controls and were considerably larger due to abundant, strongly basophilic and pyroninophilic cytoplasm. This increased fibroblastic activity was apparent in all three layers of the dermis. The fibroblasts also exhibited a marked increase in alkaline phosphatase activity, most noticeable in the cytoplasm and in the perinuclear zones, in contrast to the controls which showed only slight activity limited to the nuclei. The peripheral layer was fairly well differentiated, with proliferating capillary channels in evidence.

Sections of combs in which the intercellular ground substance was present in considerable quantities were stained with toluidine blue before and after hyaluronidase digestion. The metachromasia

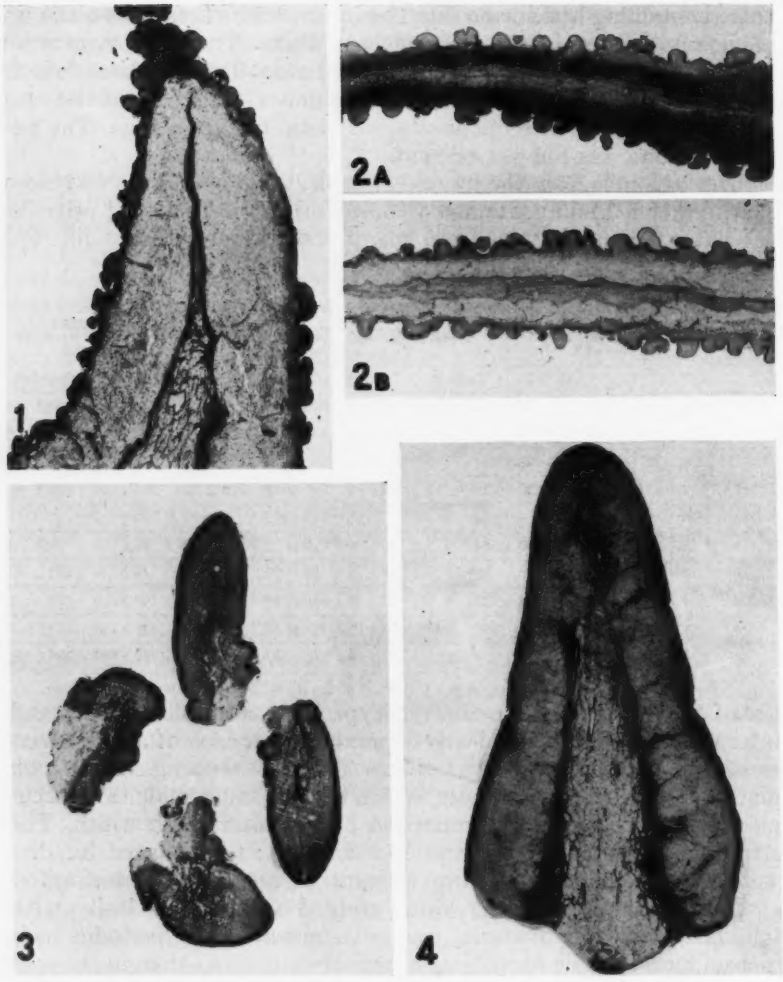

PLATE I. Figures 1 through 4, ×15.

FIG. 1. Hematoxylin and eosin. Cross section of comb of adult cock, showing sharp demarcation of central zone (core), loose intermediate zone, and the vascular subepithelial peripheral zone.

FIG. 2A. Toluidine blue stain. Portion of comb of adult cock, showing massive deposit of metachromatic intercellular ground substance in the intermediate zone.

FIG. 2B. Toluidine blue stain, following hyaluronidase digestion, of same comb. Note complete removal of metachromatic material.

FIG. 3. Hematoxylin and eosin. Comb of normal 14 day old chick.

FIG. 4. Hematoxylin and eosin. Comb of testosterone-treated 14 day old chick, revealing marked increase in size and differentiation of structural pattern.

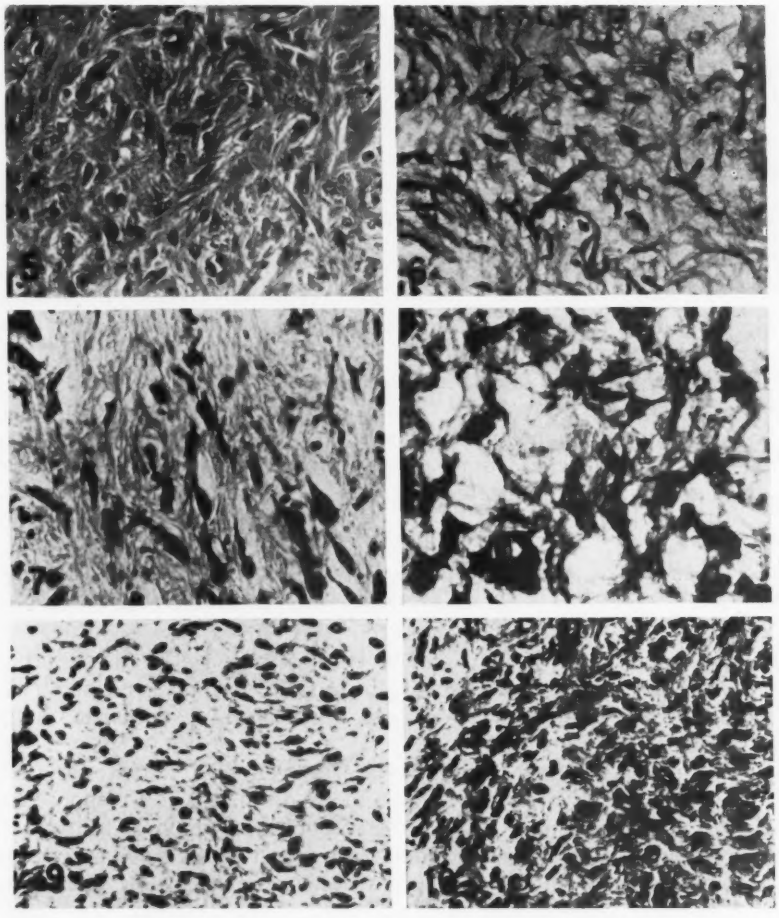

PLATE II. Figures 5, 6, 9, 10, ×660. Figures 7, 8, ×990.

FIG. 5. Toludine blue stain. Control comb. Note small fibroblasts, tightly packed collagen bundles, and absence of metachromatic ground substance

FIG. 6. Toluidine blue stain. Testosterone-treated comb. The fibroblasts are large, collagen bundles widely separated, and a finely fibrillar deposit of metachromatic connective tissue mucin is seen.

FIG. 7. Toluidine blue stain. Section from untreated chick, showing fibroblasts with scanty cytoplasm.

FIG. 8. Toluidine blue stain. Section from testosterone-treated chick to demonstrate the marked increase in the quantity and basophilia of the cytoplasm of the fibroblasts.

FIG. 9. Gomori alkaline phosphatase method, without counterstain. Comb of control chick. Only a small amount of phosphatase is visualized, primarily in the nuclei. (The necessity of using a very high contrast film exaggerates considerably the amount of phosphatase actually seen under the microscope.)

FIG. 10. Alkaline phosphatase technique. Comb of treated chick, demonstrating greatly increased concentrations of phosphatase in both nuclei and cytoplasm.

was totally removed by digestion with bull testis hyaluronidase. There was marked diminution but not complete disappearance after streptococcal hyaluronidase. Human umbilical cord sections treated and stained simultaneously behaved identically. This was highly suggestive evidence that hyaluronic acid, and perhaps other mucopolysaccharides, are present in the comb.

In Experiments 4 and 5, the animals were sacrificed at four to five day intervals up to the age of 24 days. It was consistently found that there was a sudden increase in comb size in the untreated animals after 10 to 14 days of age, indicating the onset of endogenous androgen production. This was borne out by a simultaneous spurt in testicular weights at this age. Histological examination of combs after this period revealed differences between control and androgen treated chicks which were quantitative rather than qualitative. All combs revealed changes resembling those described above for the treated animals and approached in varying degrees the adult comb structure. In every case, however, combs of the testosterone treated chicks were larger and more differentiated than those of the untreated controls.

DISCUSSION

These experiments show that the growth, differentiation, and mucopolysaccharide content of the cock's comb are dependent on androgen stimulation. This confirms the earlier investigations of Champy and Kritch (1925) and of Hardesty (1931) but is the first report of the histological effects of pure male hormone, inasmuch as the previous workers used testis implants or crude testicular extracts to stimulate comb growth.

The cellular changes observed are, in general, characteristic of young and actively growing tissues and are often seen as a response to endocrine stimulation. Cytoplasmic basophilia, which is found almost universally in the cells of such tissues, and which is so prominent in the fibroblasts of the androgen treated combs, is now generally accepted as a reflection of the amount of ribosenucleic acid present (Brachet, 1940, 1942; Dempsey and Wislocki, 1946). There has also been wide agreement that pyroninophilia is indicative of the presence of this nucleic acid, although recent evidence proves this to be less specific than formerly thought (Pollister and Leuchtenberger, 1949). The concentration of ribosenucleic acid is, in turn, correlated to the metabolic activity of the cell, and particularly to protein synthesis (Brachet, 1940, 1942; Caspersson, 1947). Brachet (1942) expresses the opinion that a high degree of cytoplasmic basophilia is related to the capacity for cell proliferation and protein formation. Caspersson (1947), using ultraviolet absorption spectrophotometry to measure ribosenucleic acid concentration, also believes that this substance is essential to protein synthesis.

The very marked increase in alkaline phosphatase content which

was seen in the treated combs is probably also a manifestation of heightened cellular metabolism. There is some evidence that the concentrations of ribosenucleic acid and alkaline phosphatase parallel one another (Kamell and Atkinson, 1948; Moog, 1946), and the suggestion has been made that they are mutually interdependent (Moog, 1946). The increase in size and number of fibroblasts, and their more pronounced mitotic activity, tend further to confirm the impression that hormone administration has stimulated the metabolic processes of these cells.

The deposition of the mucoid intercellular ground substance of the comb is also dependent on androgen stimulation and may be considered a response more specific for this tissue than the fibroblastic changes. In the untreated immature chick, no mucopolysaccharide is seen until the animal is 10–15 days of age, coinciding with the sudden spurt in testis weight seen at that time. This has been interpreted as indicating that the onset of endogenous androgen production is responsible for the appearance of the mucoid material in the comb of the cockerel. In the castrate male this material is resorbed, only to reappear when androgens are administered.

Efforts to study the nature of the ground substance of the comb revealed that a considerable proportion of the material is hyaluronic acid. The results of hyaluronidase digestion strongly suggested this, particularly since the tissue behaved so much like human umbilical cord in staining reaction and response to enzyme digestion. The specificity of these enzyme reactions has not yet been satisfactorily demonstrated, although it is possible that the streptococcal hyaluronidase, known to be more specific than the enzyme derived from the bull testis, does not react on substrates other than hyaluronic acid. Since the digestion experiments could not be accepted as providing conclusive evidence of the existence of this material in the comb, one of us extracted from this tissue a pure substance which was chemically identified as hyaluronic acid (Boas, 1949). That there are constituents other than hyaluronic acid in the ground substance of the comb cannot be doubted. A protein component, either in a free state or in chemical combination with the polysaccharides, is almost certainly present, but unfortunately cannot easily be studied by current histological or histochemical techniques.

The origin of the ground substance presents a more difficult problem. The coincidence of its appearance in the intercellular spaces with the signs of increased fibroblastic metabolic activity suggests strongly that these cells are involved in the elaboration of the ground substance. It was invariably apparent that the metachromatic material first appeared only in those portions of the combs where the fibroblasts were large and numerous and was deposited in close approximation to these cells. However, no intracellular metachromatic material was seen.

It is worth emphasizing that the deposition of hyaluronic acid and other components of the ground substance are dependent on androgen stimulation in the tissue chosen for these experiments. The cock's comb may be regarded as a target organ with the capacity to respond to this specific hormonal stimulus in this way. Other tissues have been observed to react in a very similar manner to other hormones. The sex skin of the rhesus monkey has been reported to develop a similar histologic picture, with increased fibroblastic activity and ground substance deposition, after estrogen stimulation (Aykroyd and Zuckerman, 1938). Selye (1944) has also used estrogen to produce the same response in the skin of the rhino mouse. Watson (1949) has shown that similar changes take place in pretibial myxedema, possibly due to thyrotropic hormone stimulation. The hormones under these circumstances apparently accelerate or magnify tendencies already inherent in specific tissues.

SUMMARY AND CONCLUSIONS

The growth, differentiation, and mucopolysaccharide content of the cock's comb are dependent on androgen stimulation.

Hyaluronic acid has been identified as comprising a considerable proportion of the mucopolysaccharide component of the intercellular ground substance of the comb.

The metachromatic intercellular ground substance appears only in the vicinity of growing, metabolically active fibroblasts. There is, however, no conclusive evidence that the fibroblast secretes this material.

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THE MECHANISM OF ESTROGEN INHIBITION OF COMB GROWTH IN THE COCKEREL, WITH HISTOLOGIC OBSERVATIONS[1]

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IT HAS BEEN established that androgens stimulate comb growth in the capon and in the young chick. It has also been shown that in the chick this striking growth response is due in large part to the deposition of considerable amounts of a mucinous intercellular ground substance (Ludwig and Boas, 1950). It has been further demonstrated that an important component of this ground substance is hyaluronic acid (Boas, 1949).

Estrogens, on the other hand, invariably suppress normal comb growth in the young chick (Parkes and Emmens, 1944). Despite many reports on this phenomenon, little attention has been paid to the histological changes induced by estrogens. In view of our observations that testosterone enchances the deposition of ground substance in the comb, it became necessary to examine the histological changes produced by estrogens, a comb growth-inhibiting agent.

It has been claimed that the effect of androgens and estrogens on the comb represent true sex hormone antagonism (Muhlbock, 1939). Others have stated that estrogens, rather than antagonizing androgens, suppress pituitary gonadotropin production and thereby suppress gonadal androgen production (Breneman, 1942). Finally, it has been suggested that both mechanisms are in operation (Hoskins and Koch, 1939).

Because of the marked effect of estrogens on the ground substance of the comb, it was necessary to clarify this reaction as either a local tissue response to the hormone or an indirect one mediated through its effect on either the testes or pituitary. With this in mind a series of experiments were set up which indicate clearly that estrogens inhibit comb growth only indirectly by suppressing pituitary gonadotropin production.

METHODS

Single-combed White Leghorn cockerels were used for the experiments. They were kept in a battery brooder, fed a stock chick starter mash (Quaker

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[1] Supported by a grant from the Life Insurance Medical Research Fund.

[2] Charles Klingenstein Fellow.

Oats Company) and water ad libitum. They were divided into control and treated groups of approximately equal size. At the conclusion of each experiment the chicks were sacrificed by etherization. The combs were removed according to a standard technique described by Frank, *et al.* (1942). The combs and testes were weighed on a Roller-Smith Torsion Balance and fixed in formalin and 100% alcohol for histological study. The tissues were sectioned at 5 micra and stained with hematoxylin and eosin, toluidine blue, methyl green-pyronine, and by Gomori's alkaline phosphatase technique without counterstain.

RESULTS

Experiment I. The Effect of Estrogen. One group of chicks received daily topical applications of α-estradiol[3] (75γ in 0.02 ml. of sesame oil) on the comb. The control group received only sesame oil (0.02 ml.). Half of the animals were sacrificed after 6 days of treatment (ninth day of life) and the remainder after 15 days of treatment (eighteenth day of life).

The chicks sacrificed after six days exhibited some differences between the control and the treated groups. This was not strikingly evident in the comb weights but the comb-body weight ratios (a standard correction factor) indicated inhibition of comb growth. The testes of the chicks given estrogen were significantly smaller than those of the controls (see Table 1).

TABLE 1

	Age (days)	Number chicks	Dose	Body weight (grams)	Comb weight (mg.)	Testis weight (mg.)	$\frac{CW}{BW}\times 100$
C	9	8	0	86 ± 10*	35 ± 9	30 ± 10	43 ± 7
E		9	75	91 ± 7	28 ± 9	13 ± 4	30 ± 8
C	18	8	0	156 ± 9	345 ± 144	61 ± 20	220 ± 87
E		9	75	167 ± 15	74 ± 36	26 ± 5	44 ± 19

C = Control.
E = Estrogen treated.
* $SE = \sqrt{\frac{\Sigma\Delta^2}{n(n-1)}}$.

Histologically, these combs were almost indistinguishable from each other. They were small and poorly differentiated. The fibroblasts were small and relatively sparse. The collagen bundles were tightly packed in a compact orderly fashion. No metachromatic material was seen. Alkaline phosphatase activity was minimal (Figures 1 and 2).

The animals killed at eighteen days of age exhibited more marked differences between the treated and untreated groups. Body weights

[3] α-Estradiol and testosterone generously supplied by Ciba Pharmaceutical Products, Inc.

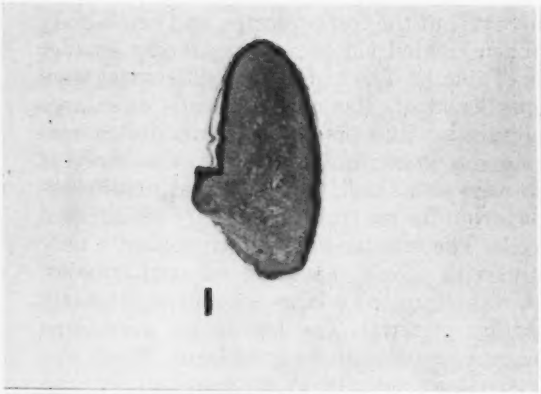
were not significantly different; but the combs, testes, and comb-body weight ratios of the estrogen treated chicks were markedly smaller than those of the controls (Table 1). The histological differences were also quite marked (Figures 3 and 4). The control combs were large and showed definite differentiation into the layered structure characteristic of the adult cock's comb, in contradistinction to the combs of the treated chicks, which were still small, compact, and undifferentiated. The core (central layer) of the controls was sharply demarcated with well developed fat cells. The intermediate layer revealed a very loose reticular architecture, with widely separated collagen bundles, in the interstices of which was deposited a large amount of intensely metachromatic, finely fibrillar material. The fibroblasts were large with abundant basophilic and pyroninophilic cytoplasm. There was considerable alkaline phosphatase activity in both cytoplasm and nuclei, most marked in the perinuclear zone. Proliferation of vascular channels in the peripheral layer was also present.

The estrogen treated combs still retained the immature structure seen in the combs of the younger chicks. There were no clearly demarcated layers but an undifferentiated dermis of compact dense collagenous bundles in which were interspersed small poorly staining fibroblasts. No metachromasia was present.

Experiments II and III. The Effects of Estrogen-Gonadotropin Mixtures. These experiments may be considered together. In both, the animals were divided into four groups as follows: (a) untreated controls, (b) those receiving estrogen alone, (c) those receiving estrogen and gonadotropin, and (d) those given gonadotropin alone. The sole difference between the two experiments lies in the dosage of gonadotropin. In Experiment II 30 IU of gonadotropin[4] (pregnant mare's serum) were injected daily, and in Experiment III 9 IU of the same material was used. For those animals receiving estrogen, 50 gamma of α-estradiol dissolved in 0.02 ml. of sesame oil were applied topically to the comb. Hormone administration was started on the third day of life, and the chicks were sacrificed after five days of treatment.

None of the groups showed any important variation in body weights (Table 2). Those given estrogen alone demonstrated no significant difference from the control group in comb weights or comb-body weight ratios. However, definite inhibition of testis growth was apparent in the chicks receiving estrogen. The groups treated with gonadotropin alone and with gonadotropin plus estrogen showed no significant variation from each other in comb weight, comb-body weight ratio, or testis weight, but gave distinctly larger values for these factors than the control or estrogen treated groups. It is also worth noting that comb and testis weights were greater in the animals given 30 IU of gonadotropin than in those getting 9 IU, sug-

[4] Gonadogen, manufactured by Upjohn.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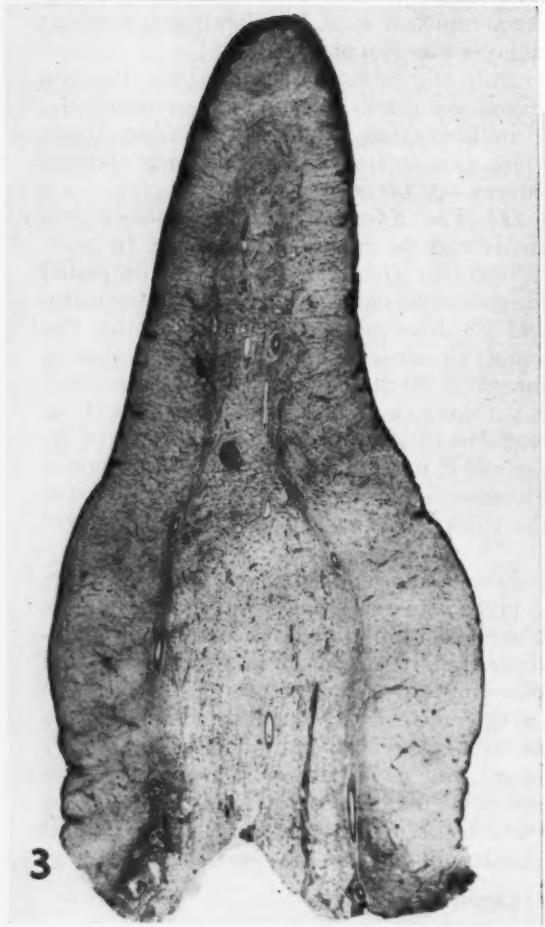
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TABLE 2

	Number chicks	Dose	Body weight (grams)	Comb weight (mg.)	$\frac{CW}{BW} \times 100$	Testis weight (mg.)
EXPERIMENT II	10	*G 0 †E 0	64 ± 6	24 ± 5	37 ± 6	20 ± 5
	10	G 0 E 50	66 ± 9	20 ± 4	30 ± 5	10 ± 2
	10	G 30 E 50	72 ± 4	57 ± 18	79 ± 25	64 ± 18
	8	G 30 E 0	70 ± 5	53 ± 14	74 ± 15	64 ± 19
EXPERIMENT III	10	G 0 E 0	70 ± 7	25 ± 4	35 ± 4	21 ± 7
	9	G 0 E 50	66 ± 6	19 ± 3	29 ± 5	11 ± 3
	9	G 9 E 50	68 ± 6	41 ± 11	60 ± 17	32 ± 6
	8	G 9 E 0	71 ± 7	43 ± 17	59 ± 23	38 ± 12

* G = Gonadogen.
† E = Estradiol.

gesting a quantitative relationship between the dose of gonadotropin and the response.

Microscopically, the combs follow the pattern already described. The control and estrogen treated tissues are immature and undifferentiated. The combs from the gonadotropin treated chicks are typical of those responding to androgen stimulation, with evidences of rapid growth and differentiation and large amounts of metachromatic material.

Experiment IV. The Effects of Estrogen-Androgen Mixtures. The chicks were divided into an untreated control group, and five groups each receiving a constant dose of testosterone[3] (50 gamma) and varying amounts of estradiol. The hormones were administered in 0.02 ml. of sesame oil by direct application to the comb. Treatment was started on the third day of life and continued for seven days.

All sections stained with hematoxylin and eosin. ×15.

FIG. 1. Section of comb of 9 day old control chick, showing small size and undifferentiated structure.

FIG. 2. Section of comb of 9 day old estrogen treated chick. Note marked similarity to control.

FIG. 3. Section of comb of 18 day old control chick, with marked increase in size and differentiation into the layered structure characteristic of the adult cock's comb.

FIG. 4. Section of comb of 18 day old estrogen treated chick, demonstrating retention of small size and immature morphology.

At the conclusion of the experimental period, the body weights of all six groups were within the same range (Table 3). All chicks receiving testosterone demonstrated a marked increase in comb weight and comb-body weight ratios and a definite decrease in testis weight in comparison to the control group, but were not significantly different from each other regardless of whether the androgen was given alone or in combination with estrogen. Testis weights were significantly reduced in all groups except the controls; it is interesting to note that testosterone alone was as effective as estradiol in effecting this reduction.

TABLE 3

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
	Sesame oil control	*T—50 gamma †E— 0	T—50 gamma E— 5 gamma	T—50 gamma E—25 gamma	T—50 gamma E—50 gamma	T— 50 gamma E—100 gamma
No. of animals	12	9	11	11	10	11
Body weight (grams)	88 ±2	85 ± 3	84 ± 3	81 ± 3	89 ± 2	86 ± 4
Comb weight (mg.)	49 ±3	231 ±19	216 ±12	231 ±12	236 ±19	205 ±22
$\frac{CW}{BW} \times 100$	56 ±4	272 ±21	257 ±12	289 ±22	274 ±26	236 ±21
Testis weight (mg.)	32.4±1.9	16.7± 1.8	18.2± 1.9	15.9± 2.4	16.2± 1.0	15.2± 1.2

* T—Testosterone.
† E—Estradiol benzoate.

DISCUSSION

The administration of estradiol to young male chicks inhibits the normal growth and development of the comb. Estradiol suppresses the sudden spurt in comb sizes normally seen between 10 and 14 days of age which is due to the onset of endogenous androgen production. The combs remain small and retain the relatively undifferentiated structure characteristic of the newborn chick. There is no visible deposition of interstitial ground substance. The histologic picture is thus, in a sense, a negative one, since it consists of an absence of changes rather than specific findings ascribable to the action of estrogen. It was therefore necessary to resort to means other than direct observations of alterations in comb structure to clarify the mechanism of estrogen inhibition of comb development.

The growth and maintenance of the male comb structure is initiated and sustained by constant stimulation by androgens. Since estrogens block this effect their mechanism of action falls into four possible categories, namely, (1) a direct local inhibitory effect on the comb tissues themselves, (2) a neutralizing or antagonistic influence on the androgens peripherally, (3) a suppression of the secretion of androgens by direct action on the testis, or (4) inhibition of gonadotropin production by the adenohypophysis.

If estrogens exerted a direct inhibitory influence on the tissues of the comb or manifested a true antagonism to androgens, one would

expect that they would produce some diminution of the growth induced by testosterone (Experiment IV) or gonadotropin (Experiments II and III). However, the comb weights of chicks given either of these substances reveal that the simultaneous administration of estrogen did not modify the response. This was further confirmed by the very marked similarity of the histological findings in the combs from such animals. That the comb shows no loss of ability to react to androgen stimulation in the presence of estradiol indicates that estrogens have neither a local inhibitory effect on the comb tissues nor a peripheral antagonistic influence. Therefore the absence of normal comb growth in chicks treated with estrogen alone must be ascribed to diminished androgen secretion.

That the site of action is not the testis is apparent from the data in Experiments II and III. The increased testis weights in the gonadotropin treated chicks is evidence of a response to gonadotropic stimulation. Here again the administration of estradiol failed to diminish the effect, indicating that estrogen does not impair the capacity of the testis to react even to small doses of gonadotropin.

Thus, of the four possible pathways suggested above, the first three are unlikely in view of the results of these experimental studies. In addition the findings offer positive support for the suppression of gonadotropin formation in the adenohypophysis. In all animals given estrogen alone, the testes were significantly smaller than those of the control chicks. Since the ability of these organs to respond to gonadotropic stimulation is not reduced, one must assume that there has been a diminution of gonadotropin output probably as a result of estrogenic depression of adenohypophyseal function.

This conclusion is in agreement with many, but not all, of the previous reports on the subject. Juhn, D'Amour, and Womack (1930) injected combinations of androgens and estrogens into capons and produced simultaneous comb growth and female plumage. They suggested that this demonstrated the absence of antagonism or syngerism between male and female hormones. Kosin and Munro (1942) using male chicks as test animals also found no interference with androgen-stimulated comb growth by estrogens. Payne (1942) reported that estrone produced a marked diminution in the number of basophil cells of the pituitary. Breneman (1942) again studying cockerels felt that the inhibitory action of estrogen on the comb was mediated through the pituitary. Pfeiffer (1947) reported that in the sparrow, in which gonadotropin secretion is limited to the period preceding the breeding season, estrogens brought about a reduction in testicular size only when given during this period and was without effect at other times.

On the other hand Muhlbock (1939) gave male and female hormones simultaneously to capons and reported partial or complete suppression of the usual androgen response in the comb. However,

he used minimal doses of testosterone and relatively enormous doses of estrogen and presented no actual data other than overall percentile changes in comb size, so that critical evaluation is impossible. Morato-Manaro and Albrieux (1939) reached similar conclusions but on the basis of the study of only two capons, without controls. Hoskins and Koch (1939) also treated capons with both androgens and estrogens and claimed that the comb growth response was less than with androgen alone.

These papers offer some support for the concept of true androgen-estrogen antagonism in relation to the growth of the cock's comb. It is difficult to reconcile these reports with those of the other investigators, particularly since the results of our own experiments, using previously untried methods, i.e., gonadotropin and histologic studies, conform to one pattern suggesting that the sole mechanism of comb-inhibition by estradiol is by way of the adenohypophysis.

SUMMARY

The administration of α-estradiol to immature male chicks prevents the normal growth and development of the comb. Histological examination of the combs of estrogen-treated birds revealed the failure to deposit the metachromatic interstitial ground substance normally seen and the persistence of the immature undifferentiated structure.

α-Estradiol failed to diminish the comb growth response produced by testosterone and gonadotropins, indicating that estrogenic inhibition of comb growth is dependent on the suppression of gonadotropin secretion in the adenohypophysis.

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RENAL FACTORS IN THE ADRENAL CORTICAL CONTROL OF WATER METABOLISM[1,2]

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IT IS NOW well established that (a) the diuretic response to water is sub-normal or lacking in either animals or men with adrenal insufficiency; (b) that this deficiency is peculiarly refractory to replacement therapy; (c) that there is a susceptibility to water intoxication following adrenalectomy, due to extra-renal as well as renal abnormalities; and (d) that water diuresis is stimulated in normal animals by administration of hormones of the adrenal cortex. The mechanisms involved in these phenomena are far from clear. The pertinent specific literature has been reviewed by Levy, Marschelle and Kepler (1946); Gaunt (1946); Kendall (1948); Gaunt, Birnie and Eversole (1949).

The present study is an attempt to determine the importance of variations in glomerular filtration and tubular reabsorption of water in (a) adrenal deficient animals receiving diuretic or intoxicating doses of water, (b) in adrenalectomized animals treated with adrenal steroids, and (c) after overdosage with adrenal steroids in intact animals.

While an extensive literature exists concerning the effects of adrenal hormones on renal function, the relation of most of it to the problem considered here is not clear. In instances where the experimental situations were similar, our results confirm and extend those of Gersh and Grollman (1939), Winter and Ingram (1943), Friedman, MacKenzie and Friedman (1948), Lotspeich (1949), and Roemmelt, Sartorius and Pitts (1949).

Abstracts of part of this work have been published (Boss, Birnie and Gaunt, 1949a; 1949b).

MATERIAL AND METHODS

Mature hooded female rats weighing approximately 200 grams were used in all experiments. They were fasted for 18 hours prior to testing, but were allowed water. At the termination of the fasting period, doses of

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distilled water amounting to 3 ml. per 100 sq. cm. of body surface were warmed to 100°F. and administered by stomach tube. The dose for a 200 gram rat was 9 ml. with the body surface calculated according to the formula of Benedict (1938). Unless otherwise specified, two doses of water at hourly intervals were administered.

Creatinine clearance was used as a measure of the rate of glomerular filtration, the technique being essentially that employed by Lotspeich (1949) but modified in the volume of water administered. With practice, it was possible to express the urine satisfactorily at the collecting periods with no evident painful excitation of the animals and this perhaps accounts for the relatively high filtration rates obtained. Creatinine was injected subcutaneously at the same time that the last dose of water was given, and the clearance observation was started 30 minutes later.

The animals were fed Purina laboratory chow and were kept in animal rooms at approximately 75°F.

RESULTS

Untreated Adrenalectomized Rats. When rats which had been adrenalectomized for 18 hours and not given supportive therapy were hydrated with two doses of water at hourly intervals, the rate of glomerular filtration was depressed when compared with that of intact controls. The rate of urine flow in such adrenalectomized animals was greatly depressed (Table 1, Series A). Seven days after adrenalectomy there was a further slight decline in glomerular filtration but the rate of urine flow decreased to the extremely low figure of 0.0046 ml. per 100 gms. per minute (Table 1, Series A). The filtration rate was never depressed as much in percentage terms as was the rate of urine flow.

TABLE 1. MEAN FIGURES ON GLOMERULAR FILTRATION RATE AND URINE FLOW IN NORMAL AND ADRENALECTOMIZED RATS

Groups	Number of animals	Glomerular filtration rate ±S.E.	Urine flow ±S.E.
		ml./100 gm./min.	ml./100 gm./min.
Series A—Two hourly doses water			
Normal (control)	20	1.002 ± .0399	.0594 ± .00455
Adrenalectomized 18 hours	13	.759 ± .0332	.0237 ± .00334
Adrenalectomized 7 days	12	.701 ± .0625	.0046 ± .000728
Series B—Four hourly doses of water			
Normal (controls)	13	1.029 ± .0317	.0672 ± .00774
Adrenalectomized 18 hours	12	.590 ± .0400	.0178 ± .00402
Adrenalectomized 7 days	10	.447 ± .0803	.0035 ± .000718

When the water load was increased to four hourly doses, the expected symptoms of water intoxication (Gaunt, 1944) became evident. These included a drop in body temperature but not convulsions or death. Under these conditions a somewhat different picture of renal function was observed, one in which the decline of filtration was

a factor of great magnitude. In animals adrenalectomized for 18 hours glomerular filtration decreased more than 40 per cent below that of intact rats receiving similar treatment. This was associated with the expected marked drop in the rate of urine flow (Table 1, Series B). The decline in glomerular filtration becomes more significant in view of the fact that in normal animals given these large amounts of water there was, if any difference, a slight increase in glomerular filtration over that of those receiving less water (cf. Table 1, Series A and B). Seven days after adrenalectomy the intoxicating doses of water induced a further decline in the rate of glomerular filtration to less than half that of the intact rats; this was associated with a negligible rate of urine flow (Table 1, Series B). Thus it seems probable that while at the lower water loads the primary disturbance in renal function is an increase in tubular reabsorption, at the higher water loads the picture is complicated by severe reductions in glomerular filtration.

Replacement Therapy. Studies on replacement therapy (Table 2) showed that when a 1 per cent NaCl solution was given as drinking fluid to adrenalectomized rats for a period of seven days, glomerular

TABLE 2. MEAN FIGURES ON GLOMERULAR FILTRATION RATE AND URINE FLOW IN ADRENALECTOMIZED RATS RECEIVING SALINE OR ADRENAL STEROID THERAPY

Groups	Number of animals	Glomerular filtration rate ±S.E.	Urine flow ±S.E.
		ml./100 gm./min.	ml./100 gm./min.
Adrenalectomized 7 days Saline	10	.847 ± .0746	.0140 ± .00362
Adrenalectomized 7 days/DCA 0.5 mg./day	12	.844 ± .0788	.0183 ± .00342
Adrenalectomized 5 days/DCA 5.0 mg./5 days	8	.842 ± .0314	.0619 ± .00335

filtration was partially restored toward normal. This finding is in general agreement with those of Lotspeich (1948, 1949) and Friedman, *et al.* (1948). The rate of urine flow, however, did not approach normal levels under this therapy. Daily injections of 0.5 mg. of DCA, a dose which ordinarily maintains rats in apparent good health, gave effects almost identical with those of saline maintenance. These findings are consistent with previous reports (Gaunt, 1946) that ordinary replacement therapies do not permit a normal rate of elimination of administered water. It was found, however, that if 5 mg. of DCA per day was given for 5 days urine flow was returned to normal even without further elevation of glomerular filtration. This heavy therapy was the first in our experience to give full replacement in experiments of this type.

Overdosage Studies. Using intact rats (Table 3) it was found that 4 ml. of cortical extract (Upjohn) administered orally (2 ml. with each

water dose) was without effect on glomerular filtration but caused a significant increase in the rate of urine flow. Likewise, one 5.0 mg. dose of DCA given subcutaneously (1 hour prior to clearance tests) caused no increase in glomerular filtration but again there was a significant increase in the rate of urine flow. The administration of 5.0 mg. of DCA per day for 5 days to intact rats, unlike the situation in adrenalectomized animals, was no more effective than acute treatment.

TABLE 3. MEAN FIGURES ON GLOMERULAR FILTRATION RATE AND URINE FLOW IN INTACT RATS RECEIVING ADRENAL STEROIDS

Groups	Number of animals	Glomerular filtration rate ±S.E.	Urine flow ±S.E.
		ml./100 gm./min.	ml./100 gm./min.
DCA, 5 mg./1 day	12	.900 ± .0259	.0751 ± .00367
DCA, 5 mg./5 days	7	.831 ± .0333	.0761 ± .00733
Cortical Extract, 4 ml./1 day	12	.993 ± .0379	.0763 ± .00617

It is apparent in experiments of this type that the diuretic action of cortical hormones, whatever the details of the mechanisms may have been, was due primarily to an inhibition of the tubular reabsorption of water.

DCA and the Filtration Rate. It was noted that a relatively low filtration rate characterized all groups, both intact and adrenalectomized, receiving large doses of DCA. This was not true of the one series which received whole extract of the adrenal cortex. It is planned to investigate the significance of this finding; it may be associated with slight "anesthetic" actions of DCA (Selye, 1941). In any case, the fact that urine flow can be restored in adrenalectomized animals or enhanced in normal ones despite relatively low filtration rates increases the emphasis which must be given to the reabsorption factors.

DISCUSSION

A decrease in renal plasma flow and glomerular filtration has been seen by many other workers both in Addison's disease and in experimental adrenal insufficiency. This undoubtedly contributes to the depression in the diuretic response to ingested water which characterizes adrenal insufficiency and which serves as the basis for one of the common tests for Addison's disease (Robinson, Powers and Kepler, 1941). It is interesting that the decrease in filtration is greater at mildly intoxicating water loads than at moderate ones, because evidence of circulatory failure in water intoxication, although not systematically searched for, has not been found. Blood pressure at least is maintained surprisingly well (Swingle, Parkins, Taylor and Hays, 1937; Gaunt, 1944).

A lesser volume of evidence, on the other hand, has indicated or could be interpreted to indicate, that an effect on the tubular reabsorption of water, rather than on the glomerular filtration rate, was the factor of primary importance in the diuretic actions of cortical hormones (Gersh and Grollman, 1939; Winter and Ingram, 1943; Zierler and Lilienthal, 1948; Lotspeich, 1949; Roemmelt *et al*, 1949).

The following results from our experiments strongly support this view: (a) in untreated adrenalectomized rats the filtration rate was reduced proportionately less than the urine flow; (b) in replacement experiments chronic treatment with large doses of DCA restored urine flow to normal despite a slightly subnormal filtration rate; and (c) in normal animals both DCA and adrenal cortical extract enhanced diuresis without elevating the filtration rate.

The accumulation of antidiuretic substances resembling pituitary ADH in the urine (Martin, Herrlich and Fazekas, 1939) or blood (Birnie, Jenkins, Eversole and Gaunt, 1949) of adrenalectomized animals and Addisonian patients (Lloyd and Lobotsky, 1949), or an enhanced sensitivity to such substances after adrenalectomy (Silvette and Britton, 1938; Birnie, Jenkins, Eversole and Gaunt, 1949), makes an increased tubular reabsorption of water not only understandable but expected.

There is no evidence, however, to indicate that a reduction in the circulating levels of antidiuretic substances following overdosages of cortical hormones causes the decreased reabsorption of water and increased diuresis that follows such treatment (Eversole, 1949). There is other evidence to indicate that factors operative in depressing diuresis in adrenal insufficiency may not be complete counterparts of those that enhance diuresis following the administration of adrenal steroids to normal animals. Extra-renal factors, for example, are apparent in adrenal deficiency, the opposite of which have not been seen in overdosage experiments (Birnie, Eversole and Gaunt, 1948).

While it is clear that the cortical hormones are diuretic agents due in part to renal influences such as are indicated in these experiments, it is also well known that certain adrenal steroids may cause sodium retention and as a consequence cause water retention even to the extent of edema formation.

Results such as we obtained are best seen under the conditions of heavy water loads and large doses of DCA. Although clearance studies were not done, we were generally unable to stimulate diuresis above normal levels in acute tests on intact rats with DCA under the following conditions: (1) when small doses of water (2 ml./100 grams) were given; (2) when normal saline was used as a diuretic fluid, or (3) in mild dehydration (Osborn and Eversole, 1949). Under such conditions, the balance between the sodium-retaining and diuretic actions of the hormone theoretically may nullify each other or even result in antidiuresis. This is a possible, but not a certain, explanation

of the results of Sartorius and Roberts (1949) who found no diuretic effects of DCA in acute experiments in intact dogs. Somewhat different results may be expected when whole extract of the adrenal cortex is used because it is a more effective diuretic than DCA (Gaunt, Birnie and Eversole, 1949) and unlike DCA, stimulates diuresis in mild dehydration (Osborn and Eversole, 1949) and after the administration of normal saline solution. Upon chronic administration both DCA (Ragan *et al.*, 1940) and cortical extract (Ingle, 1949) cause the appearance of a diabetes insipidus-like syndrome in which increased thirst is an important feature. The background of these problems is more fully reviewed elsewhere (Gaunt, Birnie and Eversole, 1949).

SUMMARY

The rate of renal glomerular filtration, as judged by creatinine clearance, is depressed in untreated adrenalectomized rats receiving either diuretic or mildly intoxicating doses of water. The depression is greatest in animals receiving heavy water loads. In all adrenalectomized animals, however, the rate of urine flow was decreased proportionately more than the filtration rate.

In replacement experiments with saline or small doses of DCA both filtration and urine flow were elevated, but the latter to only a slight extent. Large doses of DCA restored urine flow to normal without full restortion of the filtration rate.

Overdosage of normal rats with either DCA or adrenal cortical extract enhanced the rate of urine flow without increasing filtration.

These results together with those of others are taken to indicate that, while maintenance of a normal filtration rate is obviously a function of importance, the primary action of cortical hormones on water excretion is to inhibit the tubular reabsorption of water.

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THE INABILITY OF EPINEPHRINE OR ADRENOCORTICOTROPIC HORMONE TO DEPLETE THE ASCORBIC ACID CONTENT OF THE CHICK ADRENAL[1]

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IT HAS BEEN demonstrated that the administration of adrenocorticotropic hormone (ACTH) results in an almost immediate fall in the cholesterol and ascorbic acid content of the rat and guinea pig adrenal gland (Sayers, Sayers, Fry, White and Long (1944), Sayers, Sayers, Sayers, Liang and Long (1946)). This decrease in cholesterol content reaches a maximum within 4 hours while the decline in ascorbic acid is detectable within twenty minutes and reaches its maximum within an hour after the intravenous injection of ACTH. The decrease in both these substances has been correlated with evidence of adrenal cortical secretion, e.g. increase in liver glycogen and lymphopenia. They have also shown that the exposure of normal animals to various traumatizing procedures (cold, scalding, hemorrhage, the injection of epinephrine, histamine and typhoid vaccine) resulted in a similar decrease in the concentration of cholesterol and ascorbic acid in the adrenal gland. However, in hypophysectomized rats, these procedures cause no alteration in adrenal cholesterol and ascorbic acid. It appears obvious that these changes are mediated through the release of ACTH by the pituitary gland.

Sayers, Sayers and Woodbury (1948) have utilized the phenomenon of the decrease in adrenal ascorbic acid in the hypophysectomized rat as an assay method for the detection of ACTH. They have demonstrated a linear relationship between the fall in the adrenal ascorbic acid and the amount of ACTH administered.

Bates, Riddle and Miller (1940) have previously shown that the young chick adrenals did not enlarge as a result of the administration of various noxious substances but did as a result of the administration of an admittedly crude preparation of adrenotropic hormone. Therefore, it seemed possible that the young chick might be used as a test

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animal for the assay of ACTH and thus obviate the use of hypophysectomized animals. Unfortunately neither the administration of ACTH nor epinephrine results in an appreciable decline in adrenal ascorbic acid in the young chick.

METHODS AND MATERIALS

White Leghorn cockerels were obtained within 24 hours after hatching, from a commercial hatchery (Hall Brothers, Wallingford, Conn.). They were kept in a battery brooder and fed a stock chick starter mash (Quaker Oats Co.) and water ad libitum.

In general two types of experiments were performed, acute and chronic. In the acute experiments, the chicks, at varying ages, were given either a

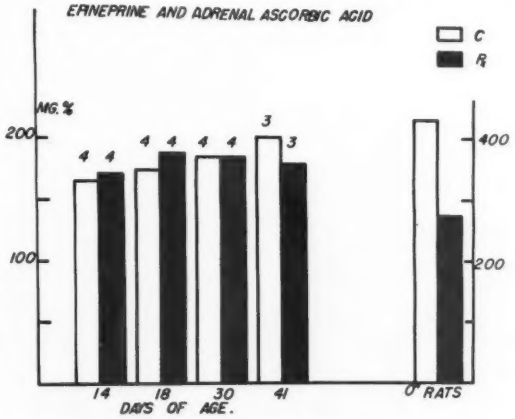

FIG. 1. The effect of epinephrine on the adrenal ascorbic acid content of the chick. The blank bars represent control values and the black, the injected. The number above the bars indicate the number of animals used. The figures on the right indicate our results on adult male rats.

single intramuscular injection of toxic but sublethal amounts (0.15–0.5 mg.) of aqueous epinephrine, or 25 mg. of ACTH (Armour)[4] and sacrificed 1 to 4 hours later. In two experiments 5–10 mg. of ACTH were injected intravenously. In the chronic experiments, 12 chicks were injected with 0.15 mg. epinephrine twice daily from the 7th to 11th day of life and killed on the 12th; another group of chicks were given 1.5 mg. of ACTH intramuscularly twice daily from the 9th to 13th days of life and killed on the 14th. In all experiments chicks from the same brood were used as controls.

In order to minimize struggling in killing the animals, the younger chicks were injected intraperitoneally with Nembutal and the older ones intravenously. The Nembutal had been previously warmed to body temperature.

The adrenals were dissected free of connective tissue and weighed on a Roller-Smith Torsion Balance. The chick's two adrenals were pooled for each determination. Ascorbic acid was determined by the method of Roe and

[4] Adrenocorticotropic hormone (ACTH) kindly supplied by Dr. John Mote of the Armour Laboratories, Chicago.

Kuether (1943). Frozen sections of the adrenals were stained with Sudan IV for sudanophilic material.

RESULTS

The ascorbic acid concentration of the newly hatched chick's adrenal averages 96 mg.%. There is a gradual rise in the concentration of adrenal ascorbic acid with age, reaching a maximum of somewhat less than 200 mg.% after 10 days of life. This is in contrast to the adrenal ascorbic acid of the rat adrenal which is above 400 mg.%.

The administration of epinephrine does not cause any appreciable change in the concentration of adrenal ascorbic acid. The results are presented in Figure 1. Animals were injected with epinephrine at the various ages indicated up to 41 days of age and in no group was there any indication of a decline in adrenal Vitamin C as a result of treatment. This is in direct contrast to the data obtained in the rat which appears on the right in Figure 1.

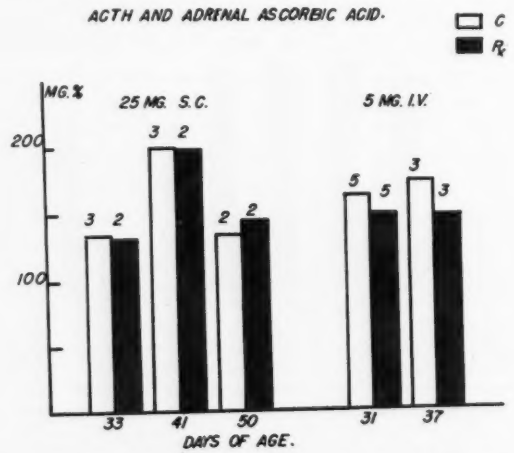

FIG. 2. The effect of ACTH on adrenal ascorbic of the chick. S.C. = subcutaneous administration. I.V. = intravenous administration.

In order to ascertain whether the adrenal or pituitary was the unresponsive factor in this phenomenon, chicks were injected at various ages with ACTH. These results are shown in Figure 2. In only one experiment was there a significant fall in adrenal ascorbic acid and that averaged only 17%.

Several animals were injected with epinephrine and their adrenals stained with Sudan IV to determine whether there was a depletion of the sudanophilic material as a result of "stress." A definite decrease in sudanophilic material occurred.

In another series of experiments young chicks were injected with either epinephrine or ACTH for 5 days in order to determine whether

the prolonged administration of these substances would cause enlargement of the adrenals. These results are shown in Figure 3. Under the conditions of this experiment, epinephrine caused a 71 per cent increase and ACTH a 26 per cent increase in adrenal weight, both of which were statistically significant. The adrenals of the latter group

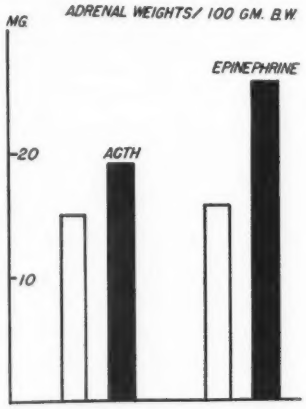

FIG. 3. The effect of daily administration of epinephrine and ACTH over a 5 day period on adrenal weight in the chick.

were also analyzed for ascorbic acid and it was found that both the control and experimental group had an average concentration of 186 mg. %.

DISCUSSION

A single injection of ACTH or epinephrine results in an immediate fall in adrenal ascorbic acid and cholesterol in the rat. Evidence has been presented by Long (1947) which indicates that these substances are in some way involved in the manufacture and/or secretion of adrenal cortical hormones. The rat is capable of synthesizing its own Vitamin C so that it is impossible to deplete its stores of the vitamin. This is not so in the guinea pig and Long (1947) has shown that although the adrenals of scorbutic guinea pigs will respond to ACTH, there is no fall in the already depleted stores of ascorbic acid. Thus, the adrenal cortical hormones can be secreted and/or synthesized without the intervention of ascorbic acid.

Since the chick adrenals hypertrophy following the chronic administration of epinephrine or ACTH and since epinephrine depletes adrenal sudanophilic substances without altering the ascorbic acid content, it would seem that in the chick, decrease in adrenal ascorbic acid is not a necessary concomitant to the release of corticosteroids. The chick, like the rat, but unlike the guinea pig, can synthesize its own Vitamin C (Carrick and Hauge (1925)).

It appears possible in view of the results of Block and Rittenberg

(1942) that cholesterol is converted to at least some of the adrenal steroids. The function of ascorbic acid in this mechanism is obscure. Lowenstein and Zwemer (1942) claimed to have isolated a steroid from the adrenal containing ascorbic acid in the molecular moiety. This has not been confirmed as yet.

SUMMARY

Chronic administration of epinephrine or ACTH to the young chick results in hypertrophy of the adrenal gland.

Neither epinephrine nor ACTH as an acute stress were capable of altering the adrenal ascorbic acid content under the conditions of this experiment. Epinephrine, on the other hand, is capable of depleting the adrenal sudanophilic material. This seems to indicate that, contrary to experience with the rat and guinea pig, the release of corticosteroids from the chick adrenal is not dependent upon a fall in the adrenal ascorbic acid.

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THE ANTIDIABETIC ACTION OF INSULIN ON NITROGEN METABOLISM[1]

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NUMEROUS reports have appeared concerning the effects produced on nitrogen metabolism by the administration of insulin to *normal* animals (Luck, *et al.*, 1928; Milhorat and Chambers, 1928; Kerr and Krikorian, 1929; Bischoff and Long, 1929; Daniels and Luck, 1931; Goldblatt and Ellis, 1931; Powers and Reis, 1933; Davis and Van Winkle, 1934; MacKay, *et al.*, 1939). But this method of study does not necessarily reveal the action of endogenous insulin in the normal animal. Even though the blood sugar remains normal, extra insulin, when introduced into an animal already provided with an adequate supply, may not exert the same effect as when injected into an animal deprived of its endogenous supply of the hormone. Again, should the blood sugar fall after the injection of insulin, the reactions that follow may be secondary to the hypoglycemia (e.g., epinephrine secretion) and not the result of the insulin *per se*. A typical example of the latter type of reaction was provided in a study of the effect of insulin on purine metabolism. Insulin produced a rise in the blood uric acid of the Dalmatian coach dog, but this effect was inhibited by keeping the blood sugar above normal during the action of the hormone (Chaikoff and Larson, 1935).

Atchley, *et al.* (1933), in a detailed study of the electrolyte balances in diabetic patients, had occasion to measure total nitrogen ingested and total nitrogen excreted. A negative nitrogen balance was observed in these patients when no insulin was administered, whereas the nitrogen balance became positive when diabetes was controlled with insulin.

Since the basic action of insulin consists of correcting the metabolic defects that characterize pancreatic diabetes, it seemed that the most satisfactory approach to a quantitative study of the antidiabetic action of the hormone would be obtained by using an animal completely deprived of its endogenous supply of insulin. In such an experiment, the amount of the hormone available could be accurately measured by injection, and the antidiabetic action of insulin could be deduced by comparing the reactions following insulin sufficiency with

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those following various degrees of insulin insufficiency. This procedure has been adopted in the present study.

It has long been recognized that no satisfactory constancy in the excretion of glucose and of nitrogen by completely depancreatized dogs can be readily attained with fresh, natural foodstuffs. Even though weighed amounts of such foodstuffs be used, variations in their fat, carbohydrate, and protein contents may make the daily excretion of glucose and nitrogen unpredictable. Hence, in the present investigation, a "synthetic" mixture was devised which, when fed in weighed amounts, provided (over a period of several months) a constant daily intake of calories, proteins, carbohydrate, salts, and vitamins. The animals were maintained under rigidly controlled conditions, the insulin dosage being the only factor that was varied throughout the period of observation.

EXPERIMENTAL

Following complete pancreatectomy, female dogs were fed twice daily a stock diet consisting of meat, sucrose, and bone ash. 8 units of insulin were injected at each time of feeding. Vitamin supplements (the B-complex in the form of a concentrate obtained from rice bran; A and D as cod liver oil) were added to the diet twice a week. The animals were maintained on this diet for several months before being fed the synthetic mixture. This period allowed for complete recovery from the side effects of the operation, and at the time the nitrogen studies were begun, the dogs were in excellent nutritional state.

"Synthetic" Diet Employed for Depancreatized Dogs.—The diet adopted had the following composition:

Commercial casein	60.0 per cent
Cellu flour[2]	20.0 per cent
Sucrose	14.0 per cent
Yeast	2.5 per cent
Bone ash	2.0 per cent
Salt mixture (Karr, 1920)	1.5 per cent

The various constituents were thoroughly mixed in quantities sufficient for each complete experiment. Samples from various parts of the mixture were tested for uniformity by repeated nitrogen determinations. While on the synthetic diet, the dogs were fed carefully weighed amounts of this mixture at exact 12-hour intervals, at which times the insulin was also administered. Five ml. of cod liver oil were always added to the morning meal to supply vitamins A and D.

Since the study required daily feeding as well as analyses over a period of several months, great care was exercised in the selection of animals. Only when depancreatized dogs showed a ravenous appetite for the meat diet were they selected for preliminary tests. Several animals refused the synthetic mixture partly or totally although they would immediately ingest any form of meat diet substituted for the synthetic mixture. Of a large number of dogs, two were retained since they showed a definite liking for the synthetic mixture for a long period, and immediately ingested the total quantity

[2] Supplied by the Chicago Dietetic Supply House.

placed before them. The dogs were kept in metal metabolism cages and at no time exposed to marked variation in ambient temperature.

The dogs were catheterized just before the morning meal was fed. Twenty-four-hour samples of urine were analyzed. The methods employed for nitrogen determinations of urine and feces have been noted elsewhere (Larson and Chaikoff, 1937). Creatine and creatinine were determined by the method of Folin (1914), and glucose was determined by the method of Shaffer and Somogyi (1933). Rest nitrogen is the calculated difference between the urinary total nitrogen and the sum of the urinary nitrogen components occurring as urea, ammonia, creatine, and creatinine. It includes amino acids, taurine, uric acid, etc.

RESULTS

The results obtained in 3 experiments, in which 2 depancreatized dogs were employed, are shown in Tables 1, 2, and 3. In each case, the animal received the synthetic diet, along with 8 units of unmodified insulin, twice daily, for periods varying from 3 to 8 weeks before urine analyses were begun. Following this, the urine was analysed daily for a period of 2 to 3 months. Throughout the latter period of observation, the daily treatment of the dogs, including times of feeding, insulin injection, and catheterization, was carefully standardized and

TABLE 1. THE INFLUENCE OF INSULIN ON NITROGEN AND GLUCOSE EXCRETION BY DEPANCREATIZED DOG A, FIRST RUN

Date	Insulin injected per day*	Weight of dog	Urine						
			Glucose	Total nitrogen	Urea nitrogen	Ammonia nitrogen	Creatine nitrogen	Creatinine nitrogen	Rest nitrogen
	units	kg.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Feb. 7	16	8.3		9.61	7.25	1.84	0.016	0.086	0.42
Feb. 8	16	8.3	1.33	9.90	7.66	1.65	0.017	0.085	0.49
Feb. 9	16		2.00	9.73	8.05	1.11	0.019	0.088	0.46
Feb. 10	16	8.3	2.16	9.97	8.27	1.02	0.022	0.089	0.57
Feb. 12†	8		8.21	10.02	7.31	2.15	0.028	0.085	0.45
Feb. 13	8		10.50	10.80	7.70	2.65		0.093	
Feb. 14	8	8.1	16.7	10.70	7.78	2.39	0.025	0.085	0.42
Feb. 15	8	8.1	18.9	10.40	7.69	1.80	0.024	0.088	0.80
Feb. 16	8	8.0	14.0	10.60	8.14	1.71	0.015	0.091	0.64
Feb. 17	8	8.0	15.0	10.80	8.74	1.35	0.019	0.090	0.60
Feb. 18	8	7.9	19.1	10.40	8.42	1.34	0.023	0.090	0.53
Feb. 19	8	7.9	19.3	10.87	8.80	1.32	0.020	0.090	0.64
Feb. 20	8	7.9	14.4	10.37	7.56	2.07	0.029	0.085	0.63
Mar. 6	8	7.9	17.4	10.30	8.38	1.40		0.084	
Mar. 7	8	7.9	13.2	10.45	8.52	1.34	0.020	0.084	0.49
Mar. 8	8		18.3	10.30	8.28	1.33	0.025	0.079	0.59
Mar. 9	8		17.3	10.75	8.80	1.33	0.033	0.085	0.50
Mar. 10	4	7.7	31.4	12.42	10.25	1.54	0.040	0.095	0.50
Mar. 11	4		30.6	12.12	10.05	1.31	0.031	0.089	0.64
Mar. 12	4		35.0	12.68	10.45	1.41	0.019	0.084	0.72
Mar. 13	16	7.5	8.09	10.26	8.58	1.22	0.027	0.086	0.35
Mar. 14	16	7.6	3.21	10.36	8.32	1.28	0.032	0.086	0.64
Mar. 15	16	7.6	3.72	9.60	7.61	1.36	0.027	0.085	0.52
Mar. 16	16	7.7	8.60	10.32	8.22	1.41	0.027	0.082	0.58
Mar. 17	16	7.8	4.87	10.25	8.24	1.63	0.026	0.087	0.27
Mar. 23	16		2.68	9.42	7.72	1.11	0.033	0.085	0.47
Mar. 24	16		1.58	9.50	7.58	1.25	0.026	0.081	0.56
Mar. 25	16	7.8	2.92	9.68	7.74	1.18	0.022	0.083	0.66
Mar. 26	16	7.8	2.12	9.35	7.48	1.25	0.026	0.087	0.51

Diet: 130 gm. synthetic mixture per meal fed twice daily at 9:15 A.M. and 9:15 P.M. 5 ml. cod liver oil were added to the morning meal.

Dietary nitrogen: 8.25 gm. nitrogen per 100 gm. synthetic mixture. Since the dog received 260 gm. mixture per day, the total nitrogen intake was 21.45 gm. per day.

The animal was placed on the synthetic mixture December 10, two months before the run was started.

On each of the days which have been omitted from the consecutive scheme of this table the insulin dose was the same as that for the other days of the group within which it would be included.

* Half of the insulin was injected with each meal. Unmodified insulin was used.

† Insulin changed to 8 units per day beginning with the afternoon meal of February 11.

TABLE 2. THE INFLUENCE OF INSULIN ON NITROGEN BALANCE IN DEPANCREATIZED DOG A, SECOND RUN

Date	Insulin injected per day*	Weight of dog	Urine							Nitrogen excreted in urine and feces†	Nitrogen balance‡
			Glucose	Total nitrogen	Urea nitrogen	Ammonia nitrogen	Creatine nitrogen	Creatinine nitrogen	Rest nitrogen		
	units	kg.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Apr. 28	16	7.9	7.40	10.99	8.87	1.58	0.018	0.085	0.44	21.30	+1.66
Apr. 29	16		3.96	10.71	8.72	1.27	0.020	0.081	0.62	21.02	+1.94
Apr. 30	16		7.80	11.12	8.97	1.70	0.024	0.081	0.35	21.43	+1.53
May 1	16	7.9	4.00	10.70	8.82	1.28	0.024	0.081	0.50	21.01	+1.95
May 2	16		9.40	10.90	8.74	1.43	0.021	0.080	0.63	21.21	+1.75
May 3	16		6.80	11.30	9.24	1.32	0.020	0.079	0.64	21.61	+1.35
May 4	16		8.00	10.95	8.90	1.28	0.018	0.082	0.67	21.26	+1.70
May 5	16		7.10	11.30	9.18	1.45	0.024	0.083	0.56	21.61	+1.35
May 6	16	8.0	6.80	11.30	9.24	1.36	0.024	0.081	0.60	21.61	+1.35
May 7	16		9.40	11.45	9.23	1.60	0.025	0.080	0.52	21.76	+1.20
May 8	16	7.9	5.30	11.50	9.10	1.78	0.027	0.079	0.51	21.81	+1.15
May 9	8		12.0	12.10	9.75	1.60	0.026	0.082	0.64	22.41	+0.55
May 10	8	7.8	17.7	12.15	9.90	1.51	0.021	0.083	0.64	22.46	+0.50
May 11	8		15.6	12.02	9.85	1.40	0.023	0.085	0.66	22.33	+0.63
May 12	8		17.0	12.05	10.10	1.42	0.023	0.077	0.43	22.36	+0.60
May 13	8		20.5	12.25	10.10	1.38	0.020	0.083	0.67	22.56	+0.40
May 14	8	7.7	12.5	12.45	10.00	1.78	0.021	0.080	0.57	22.76	+0.20
May 15	8		12.8	12.75	10.30	1.78				23.06	−0.10
May 16	8		21.3	12.40	10.30	1.34		0.082		22.71	+0.25
May 17	8	7.7	15.8	12.20	9.95	1.60		0.084		22.51	+0.45
May 18	4		23.8	13.20	10.74	1.78	0.034	0.081	0.57	23.51	−0.55
May 19	4		28.4	12.95	10.50	1.86	0.034	0.081	0.48	23.26	−0.30
May 20	4	7.6	21.6	12.65	10.30	1.74	0.041	0.084	0.49	22.96	0.00
May 21	16		6.95	10.80	8.84	1.54	0.018	0.078	0.32	21.11	+1.85
May 22	16	7.6	2.24	10.60	8.45	1.43	0.028	0.082	0.61	20.91	+2.05
May 23	16		1.71	10.55	8.40	1.54	0.021	0.084	0.51	20.86	+2.10
June 1	16	7.9	3.63	10.60	8.54	1.30	0.022	0.082	0.66	20.91	+2.05
June 13	16	8.0		10.70						21.01	+1.95
June 14	16			10.80						21.11	+1.85
June 15	16	8.0		10.70						21.01	+1.95
June 16	16			10.90						21.21	+1.75
June 17	16			11.00						21.31	+1.65
June 18	16			10.90						21.21	+1.75
June 20	16	7.9		10.90						21.21	+1.75
June 22	16			11.00						21.31	+1.65
June 23	16			10.90						21.21	+1.75
June 24	16		2.50	10.80	8.76	1.40	0.017	0.082	0.54	21.11	+1.85
June 25	16	8.0	2.35	11.20	9.00	1.57	0.027	0.083	0.52	21.51	+1.45
June 26	16		1.43	11.00	8.90	1.34	0.022	0.083	0.66	21.31	+1.65
July 1§	8			12.60						22.91	+0.05
July 2	8			12.30						22.61	+0.35
July 3	8	7.9		12.20						22.51	+0.45
July 4	8			12.40						22.71	+0.25
July 5	8	7.8		12.50						22.81	+0.15
July 6	8			12.30						22.61	+0.35
July 7	8	7.8		12.50						22.81	+0.15

Diet: 140 gm. synthetic mixture per meal fed twice daily at 7:30 A.M. and 7:30 P.M. 5 ml. cod liver oil were added to the morning meal.

Dietary nitrogen: 8.20 gm. nitrogen per 100 gm. synthetic mixture. Since the dog received 280 gm. mixture per day, the total nitrogen intake was 22.96 gm. per day.

The animal was placed on the synthetic mixture April 1, four weeks before the run was started.

On each of the days which have been omitted from the consecutive scheme of this table the insulin dose was the same as that for the other day of the group within which it would be included.

* Half of the insulin was injected with each meal. Unmodified insulin was used.

† Total fecal nitrogen was determined for several periods of 3 to 5 days. The variation in the calculated daily fecal nitrogen output for these periods was small, and an overall average of 10.31 gm. nitrogen per day is used throughout this table.

‡ Represents difference between total nitrogen intake in food and total nitrogen eliminated in urine and feces.

§ Insulin lowered to 8 units per day on June 28.

kept rigidly constant, the only variation permitted being the amount of insulin injected.

Dog A—First Run.—By the administration of 16 units of unmodified insulin per day, glycosuria was almost eliminated in dog A, only about 2 gm. of glucose being excreted per day (Table 1). The changes in glucose excretion in the animal during insulin reductions were very striking. Thus, as a result of the first reduction from 16 to 8 units per day, the glucose excretion rose from about 2 to about 16 gm. per day, while during the next interval, in which the insulin intake was reduced

TABLE 3. THE INFLUENCE OF INSULIN ON NITROGEN BALANCE IN DEPANCREATIZED DOG B

Date	Insulin injected per day*	Weight of dog	Urine							Nitrogen excreted in urine and feces†	Nitrogen balance‡
			Glucose	Total nitrogen	Urea nitrogen	Ammonia nitrogen	Creatine nitrogen	Creatinine nitrogen	Rest nitrogen		
	units	kg.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Sept. 21	16	7.0	0.60	7.93	6.44	0.91	0.017	0.057	0.51	18.25	+1.55
Sept. 22	16	7.0	0.50	7.90	6.38	1.05	0.020	0.060	0.39	18.22	+1.58
Sept. 23	16	7.0	0.40	8.00	6.60	1.05	0.020	0.055	0.28	18.32	+1.48
Sept. 24	12	7.0	1.70	8.22	6.72	1.03	0.029	0.054	0.39	18.54	+1.26
Sept. 25	12	7.0	1.30	8.60	6.80	1.14	0.020	0.060	0.58	18.92	+0.88
Sept. 26	12	7.0	1.00	8.25	6.60	1.11	0.020	0.061	0.46	18.57	+1.23
Sept. 27	12	6.9	1.00	8.45	6.80	1.11	0.024	0.060	0.46	18.77	+1.03
Sept. 28	12		1.00	8.14	6.57	1.11	0.022	0.063	0.38	18.46	+1.34
Sept. 29	12		2.05	8.40	6.85	1.13	0.021	0.068	0.33	18.72	+1.08
Sept. 30	12		2.05	8.55	6.96	1.05	0.020	0.060	0.46	18.87	+0.93
Oct. 1	12		4.60	8.72	7.20	1.11	0.023	0.060	0.33	19.04	+0.76
Oct. 2	12		5.60	8.90	7.34	1.05	0.020	0.061	0.43	19.22	+0.58
Oct. 17	12	6.8	4.68	9.30						19.62	+0.18
Oct. 18	12		5.00	9.20	7.68	0.85	0.025	0.060	0.59	19.52	+0.28
Oct. 23	12		3.00	8.94						19.26	+0.54
Oct. 24	12		5.50	9.20	7.76	0.80	0.019	0.060	0.56	19.52	+0.28
Oct. 25	8	6.7	13.3	9.82	7.98	1.15	0.023	0.057	0.61	20.14	−0.34
Oct. 26	8		12.5	10.26	8.18	1.43	0.034	0.054	0.56	20.58	−0.78
Oct. 29	8	6.6	21.1	10.20						20.52	−0.72
Oct. 30	8	6.6	22.8	10.20	8.52	1.00	0.021	0.062	0.60	20.52	−0.72
Oct. 31	8	6.6	20.2	9.99						20.31	−0.51
Nov. 1	8	6.6	20.9	9.82						20.14	−0.34
Nov. 2	8	6.6	18.2	9.68	7.62	1.41	0.037	0.060	0.55	20.00	−0.20
Nov. 4	8	6.6	16.0	9.70	7.60	1.50	0.033	0.060	0.51	20.02	−0.22
Nov. 8	8	6.6	18.4	9.40						19.72	+0.08
Nov. 9	8	6.5	20.9	9.60						19.92	−0.12
Nov. 12	8	6.4	19.1	9.85	7.78	1.50	0.033	0.060	0.48	20.17	−0.37
Nov. 13	4		29.8	10.50	8.60	1.33	0.040	0.060	0.47	20.82	−1.02
Nov. 14	4	6.4	37.0	11.05						21.37	−1.57
Nov. 15	4	6.4	38.2	10.90	9.05	1.25	0.038	0.062	0.50	21.22	−1.42
Nov. 16	4	6.4	34.5	10.50	8.64	1.35	0.035	0.059	0.42	20.82	−1.02
Nov. 17	4	6.3	31.4	10.40	8.60	1.25	0.029	0.059	0.46	20.72	−0.92
Nov. 18	0	6.2	81.0	10.90	8.65	1.60	0.040	0.075	0.54	21.22	−1.42
Nov. 19	0		78.6	12.12	9.84	1.86	0.052	0.068	0.30	22.44	−2.64
Nov. 20	0	6.2	76.2	12.30	10.30	1.54	0.061	0.065	0.33	22.62	−2.82

Diet: 120 gm. synthetic mixture per meal fed twice daily at 7:30 A.M. and 7:30 P.M. 5 ml. cod liver oil were added to the morning meal.

Dietary nitrogen: 8.25 gm. nitrogen per 100 gm. synthetic mixture. Since the dog received 240 gm. mixture per day, the total nitrogen intake was 19.80 gm. per day.

The animal was placed on the synthetic mixture September 2, three weeks before the run was started.

On each of the days which have been omitted from the consecutive scheme of this table the insulin dose was the same as that for the other days of the group within which it would be included.

* Half of the insulin was injected with each meal. Unmodified insulin was used.

† Total fecal nitrogen was determined for several periods of 2 days. The variation in the calculated daily fecal nitrogen output for these periods was small, and an overall average of 10.32 gm. nitrogen per day is used throughout this table.

‡ Represents difference between total nitrogen intake in food and nitrogen eliminated in urine and feces.

to 4 units per day, the glucose eliminated in the urine attained values between 30.6 to 35.0 gm.

During the initial period, when the dog received 16 units of insulin per day, the nitrogen excreted in the urine was 9.61–9.97 gm. per day, most of which was in the form of urea. When the insulin supplied was reduced to 8 units per day, the nitrogen excretion rose to 10.02–10.87 gm. per day. Following an interval of 26 days, during which the dog received 8 units daily, the insulin was reduced, for 3 days, to 4 units per day. As a result of this insulin reduction, the nitrogen excretion immediately rose to 12.12–12.68 gm. per day. When the administration of 16 units per day was resumed, the nitrogen excretion, after some fluctuations, fell to amounts close to the initial values. It may be noted from Table 1 that the increases in nitrogen excretion which accompanied the insulin reductions were due mainly to increases in urea excretion. The urinary excretion of creatine increased somewhat, but the quantity of nitrogen involved here is very small

compared to urea. The excretion of ammonia, creatinine, and rest nitrogen remained essentially constant.

The reduced insulin levels were maintained for a period of one month (Februrary 11 to March 12) during which the increased nitrogen elimination was accompanied by a progressive loss in weight; the total loss in weight during this month amounted to 0.8 kilos, about 10 per cent of the initial body weight.

Dog A—Second Run.—One week after termination of the first run described above, dog A was placed on the dietary regime for the second run. The new diet was prepared in the same manner as that used in the first run. The amount fed per meal was increased 140 gm.

Under the influence of 16 units of insulin per day, the glucose output varied from 3.96 to 9.40 gm. per day (Table 2). When the insulin injected was reduced to 8 and to 4 units per day, the urinary response of both nitrogen and glucose was equally as significant as that shown in Table 1. In this second run, however, fecal nitrogen was also determined to permit a study of the nitrogen balance. When the animal received 16 units per day, a positive nitrogen balance[3] was obtained. When the insulin administered was reduced to 4 units daily, the nitrogen excreted was greater than the intake. Although the nitrogen lost in urine and feces during the interval in which 8 units of insulin were administered was, in most cases, slightly less than the nitrogen ingested, it is questionable whether dog A was in positive balance at this time, since Voit (1866) has shown that a dog loses about 0.18 gm. nitrogen per day in the epidermis and hair.

Dog B.—This dog was studied for a period of 2 months, during which the daily insulin dosage was reduced from 16 to 0 units by steps of 4 units (Table 3). When this animal received 16 units of insulin, practically all its available carbohydrate was retained, less than 1 gm. being excreted. Each decrease in administered insulin was followed by a definite increase in the excretion of both glucose and nitrogen. While an average of 3.0 gm. glucose was eliminated on 12 units of insulin, the glucose output rose to averages of 18.5, 34.2, and 78.6 gm., respectively, when the insulin injected was 8, 4, and 0 units per day.

Beginning with an average daily excretion of 7.94 gm. of nitrogen while under the influence of 16 units of insulin, dog B eliminated (average values) 8.69, 9.87, 10.67, and 11.77 gm., respectively, of nitrogen when the insulin administration was lowered successively to 12, 8, 4, and 0 units. A positive balance was found only when the animal was receiving 16 and 12 units. When it received 8 units or less, a negative balance was obtained, and this was accompanied by a progressive loss in body weight.

The relation of nitrogen balance to the amount of injected insulin is shown in Figure 1. Under the particular conditions of this study, the relation was found to be approximately linear between 4 and 16 units.

[3] It should be noted that the term "balance" applies only to the difference between the nitrogen ingested and the nitrogen lost in urine and feces.

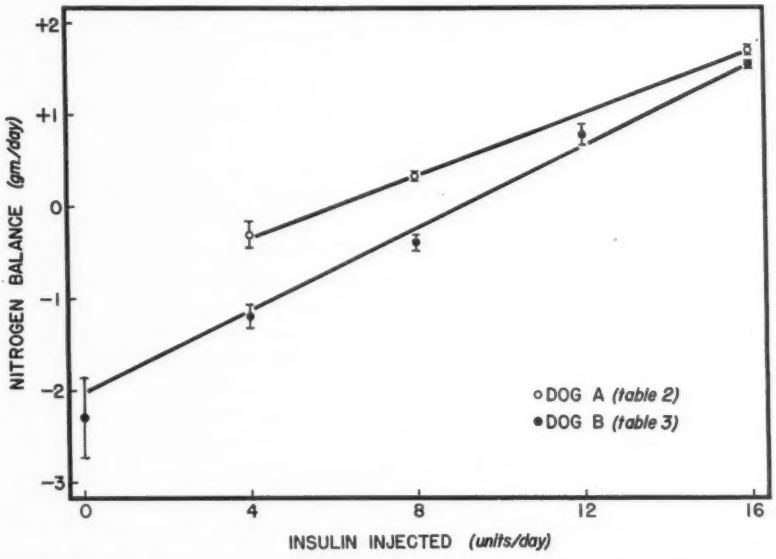

FIG. 1. The relation between nitrogen balance and the amount of insulin injected. Each point is the arithmetic mean of all nitrogen balance values obtained at the particular level of insulin administration. The vertical line-segments show the standard errors of these means.

One unit of insulin caused the retention of 0.17 gm. of nitrogen in dog A, and 0.22 gm. in dog B.[4] This relationship differs markedly from that observed for glucose excretion. In the latter case, the increase in glycosuria that accompanies a decrease in the amount of insulin injected becomes greater at the lower insulin levels, and the relation is far from linear.

DISCUSSION

The relation of insulin to nitrogen metabolism was investigated in depancreatized dogs in which the daily intake of nitrogen and other constituents was maintained constant by feeding a synthetic mixture. In animals so treated, a positive nitrogen balance could not be maintained when the administered insulin was reduced below a certain level. In both dogs, A and B, less than 8 units per day led to a negative balance, whereas with the same dietary intake, the administration of 16 units resulted in a positive balance. The response of the nitrogen metabolism of the completely depancreatized dog to alterations in insulin administration is fairly rapid, changes in nitrogen excretion appearing, as a rule, in the same 24-hour interval in which the change in the amount of insulin administered was effected.

The changes in urinary total nitrogen were due almost entirely to changes in urea. This means that the extra nitrogen eliminated by the dog receiving an insufficient amount of insulin probably had its source

[4] Calculated from the slopes of the lines in Figure 1.

in amino acids. Since the amounts of free amino acid nitrogen and other non-protein nitrogen found in the body are insufficient to account for the continued net loss of nitrogen observed here, it may be inferred that, in the absence of an adequate supply of insulin, there occurs a net loss of body protein.

Another hormone known to promote nitrogen storage in the body is the growth principle of the anterior pituitary. In view of the results obtained here on the depancreatized dog, one would not expect an animal to show the maximum nitrogen retaining effects of injected growth hormone unless an adequate supply of insulin were available. This interrelation between the growth hormone and insulin has been discussed by several investigators (Gaebler and Galbraith, 1941; Gaebler and Robinson, 1942; Frame and Russell, 1946; Russell and Cappiello, 1949; Cotes, *et al.*, 1949).

SUMMARY

The antidiabetic action of insulin has been studied in depancreatized dogs fed a "synthetic" mixture that permitted an accurate control of daily nitrogen excretion for long periods. The animals were maintained under rigidly controlled conditions except for the amounts of insulin injected.

When the quantity of insulin injected fell below a certain level, a negative nitrogen balance ensued. The quantities of nitrogen lost, when the dogs were in negative nitrogen balance, were sufficient to indicate that a net loss of body protein was involved.

Nitrogen balance was approximately linearly related to the amount of insulin injected under the particular conditions of these experiments.

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THE EFFECT OF RELATIVELY LARGE DOSES OF SESAME OIL ON OXYGEN CONSUMPTION AND THE ANTAGONISTIC ACTION OF ADRENOCORTICAL EXTRACT[1,2]

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ALTHOUGH it is generally concluded on the basis of the literature that sesame oil is physiologically inactive as a vehicle for fat soluble substances, there are indications that it is not as physiologically inactive as generally believed. Sesame oil in varying dosages has been reported to cause marked visceral damage in sarcoma bearing rats (Pollia, 1937); to shorten the mean survival time of adrenalectomized rats by 1.6 days as compared with their controls (Spurr and Kochakian, 1939); to produce toxic effects and death in normal rats as quickly as ten days after beginning the injections (Bruce and Tobin, 1940); to induce atrophy of the thymus in male rats (Clausen, 1940); to cause atrophy of the seminal vesicles and prostate gland in young mice (Crafts, 1942); and to stimulate the germinal epithelium of the ovary in young mice (Stein and Allen, 1942). It is the purpose of this investigation to show the effects on oxygen consumption of the whole organism of large doses of sesame oil and of adrenocortical extract when simultaneously administered with sesame oil.

METHODS

The common goldfish, *Carassius auratus*, was used as the experimental animal because of its poikilothermic nature and of its low class position among the vertebrates. The fish were bought from a local commercial source. They were kept from one to two weeks in large aerated aquaria prior to experimental use. The fish were fed daily on a commercial fish food preparation. All fish were fasted 12 to 16 hours prior to use in order to render the animal in a nearly "basal" state.

All determinations of oxygen consumption were made by the Winkler technique (Birge and Juday, 1911; Allee and Oesting, 1934). However, after the conclusion of the original studies in which the Winkler method was employed, the experiments were repeated with a new type of respirometer (Calhoon and Angerer). The data obtained by both methods were substantially identical within the range of experimental error.

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[2] It is a great pleasure to thank Dr. Bruce K. Wiseman, Department of Medicine, for placing a Warburg bath at our disposal.

Regular tap water was kept in a five gallon carboy from two to three days prior to use to assure a uniform gaseous concentration. The oxygen content of this water was determined prior to an experimental run (blank value). A specific fish, in the "basal" state, was placed in a 1000 ml. glass container filled with the stored tap water, and the container was hermetically sealed. After a 30 minute interval, a 200 ml. sample of water was drawn from the container and a determination of oxygen content was made (experimental value). This experimental value minus the blank value gives the amount of oxygen consumed by the specific fish during the 30 minute interval. This procedure was immediately repeated on the same fish for intervals of 60 and 90 minutes, respectively. From the data obtained for each fish a curve was plotted to show the volume (ml.) of oxygen consumed as a function of time. The value for zero time was assumed to be zero ml. of oxygen consumed. All metabolic rates were calculated on the basis of ml. of O_2 consumed/gm. wet weight of fish (in "basal" state)/hr.

RESULTS

Sesame Oil. The oxygen consumption was determined on six "normal" fish (controls) by the Winkler method. The results gave a mean value of 0.200 ± 0.011 (standard error) ml. O_2/hr./gm. at 25°C. These experiments were repeated on six different fish using a respirometer as mentioned elsewhere. This latter technique gave a mean

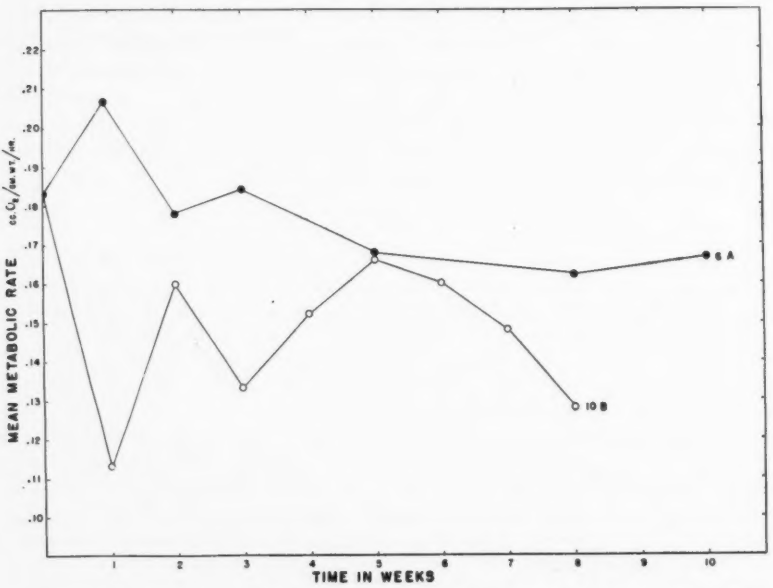

FIG. 1. Curve B shows the effect of large doses of sesame oil and Curve A the effect of simultaneous injections of large doses of sesame oil plus adrenocortical extract on the mean metabolic rates of goldfish. Both curves are adjusted to the same "normal" value. The small numerals to the right of a given curve indicate the number of fish used. Temperature ranges from 24.0°C. to 27.0°C.; pH 8.2–8.4.

oxygen consumption of 0.356 ± 0.013 ml. O_2/hr./gm. at 30°C. Although the respective means of the two sets of data obtained by the two techniques appear incongruous, they are substantially identical when their Q_{10} values are considered. Furthermore, the nature of the curves obtained by the two methods is essentially identical. These fish were given 0.0036 ml. of sesame oil/gm. of body weight intramuscularly every other day for a period of eight weeks. All determinations of oxygen consumption were made weekly and the respec-

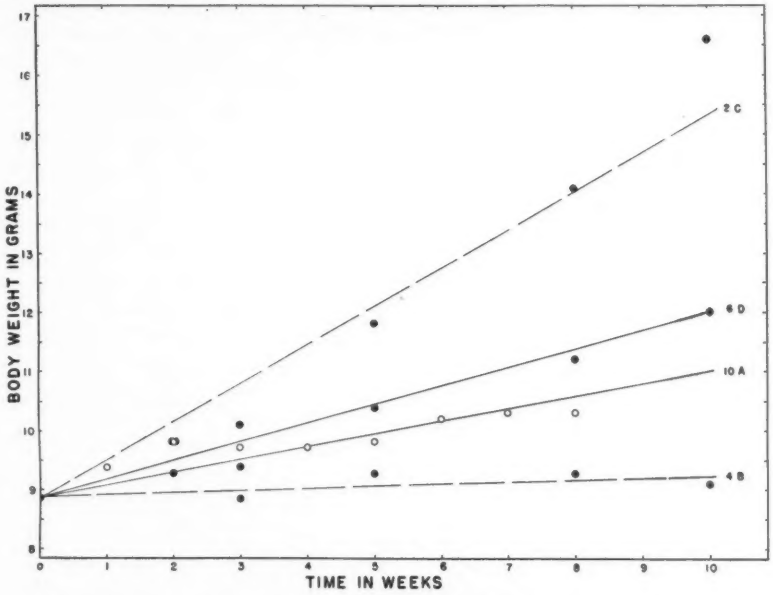

FIG. 2. Curve A shows the mean increase in body weight for all fish given large doses of sesame oil. Curve D represents the increase in mean body weight for all fish given simultaneous injections of sesame oil plus adrenocortical extract. Curve D may be broken down into two component curves representing all fish which showed an increase in body weight (curve C) and all fish which failed to give any change in body weight (curve B). All curves are adjusted to the same "normal" value. Small numerals to the right of a given curve indicate the number of fish used. Temperature ranges from 24.0°C. to 27.0°C.; pH 8.2–8.4.

tive mean values are presented in figure 1, curve B. The data show that at the end of the first week the mean oxygen consumption had decreased *ca.* 28% from the initial (control) value. Hearefter, the values show a sinusoidal character, i.e., the values for the oxygen consumption fluctuated between various maxima and minima with respect to time. The lowest mean value, *ca.* 31% from the initial (control) value, occurred at the end of the eighth week at which time the experiments were terminated.

Simultaneous Injections of Sesame Oil and Adrenocortical Extract. The "normal" (control) metabolic rates were determined on six gold-

fish using the Winkler method. Each fish was run twice thus giving a total of twelve determinations; of these, two determinations obtained from the same fish were omitted from the results because for some unknown reason they were abnormally high. The mean oxygen consumption for this group was 0.181 ± 0.011 ml. O_2/hr./g.m at 25.5°C. These animals received intramuscularly every other day for eight weeks 0.0036 ml. of sesame oil/gm. along with 0.00036 ml. of Upjohn's Adrenal Cortical Extract/gm. Again the oxygen consumptions were determined at the end of each week for a period of eight weeks and these data are presented as mean values in figure 1 (curve A). Analysis of this curve shows that at the end of the first week the mean metabolic rate had risen *ca.* 13.5%. This was followed by a slow decrease until the eighth week when the mean metabolic rate was *ca.* 11% below the initial (control) value. At the end of the first and eighth week the percentage differences between curves A and B, figure 1, are 41.5% ($P < 0.01$), and 20.0% ($P < 0.03$), respectively.

Sesame Oil and Adrenocortical Extract on Body Weight. The ten fish given sesame oil alone present a linear relation when mean body weight is plotted as a function of time (figure 2, curve A) so that at the end of the eighth week there is a weight gain of *ca.* 20% over the initial mean body weight. When the ten curves representing the weights of individual fish whose mean body weight is given in curve A are studied, eight of these curves show approximately the same slope as the mean weight curve, while each of the two remaining curves give a slight gain and a slight loss of body weight, respectively.

Curve D, figure 2, shows the mean body weight gain of all fish given adrenocortical extract along with sesame oil. This curve when compared with the curve for sesame oil (curve A) is not significant in itself because four (curve B) of the six fish used in this experiment did not gain any weight during the eight weeks interval. Actually, three of these four fish lost a slight (not statistically significant) amount of weight. The remaining two fish, (figure 2, curve C) gained weight more rapidly than fish given only sesame oil so that at the end of the eighth week a mean weight gain of *ca.* 71% over their original mean body weight was obtained. This represented a weight gain of *ca.* 51% more than those fish given sesame oil alone.

DISCUSSION

The experiments reported here show that the mean metabolic rate of goldfish given large doses of sesame oil simultaneously with adrenocortical extract remains at a value characteristic of the control, after a slight transient increase (13.5%). The mean metabolic rate of goldfish given only sesame oil decreases *ca.* 25% suggesting a kind of antagonistic action of adrenocortical extract toward the effect of sesame oil. The literature indicates that previous treatment of normal animals with adrenocortical extract tends to increase their

resistance to many forms of stress, e.g., certain drugs, infections and toxins, irradiations, shock and muscular exercise; however, in some individual instances this action is denied (Swingle and Remington, 1944; Hartman and Brownell, 1949). In line with the foregoing reports, the present study demonstrates a protection offered by adrenocortical extract against the decrease in metabolism as induced by large doses of sesame oil.

The weight curves reported here indicate that the metabolic rate does not show the complete picture. Despite the fact that the metabolic rate remained approximately normal when the fish were given adrenocortical extract plus sesame oil, the growth (assuming growth can be measured by weight gain or loss) of these fish was inhibited when given sesame oil alone. Thus, paradoxically, a situation exists where the animals are apparently protected with respect to oxygen consumption but not to body weight. Therefore, the adrenocortical extract did not offer complete protection against the effects of sesame oil. Whether it would have if the doses of sesame oil had been lighter remains to be determined.

It is shown that these dosages of sesame oil in some way make it impossible for animals simultaneously to remain normal with respect to growth and probably to metabolic processes; though, in spite of this, the adrenocortical extract can maintain, or slightly increase, oxygen consumption. Pollia (1937) showed that sesame oil injected into normal rats cause the liver, unlike other organs, to enlarge markedly, and to acquire an acetone odor. The latter suggests fatty infiltration of the liver accompanied by ketosis which would decrease the normal metabolic functions of the liver.[3] On the other hand, it must be noted that sesame oil alone did not cause an inhibition of growth which was observed when fish were given sesame oil plus adrenocortical extract. Although the oil may have impaired liver function, it is possible that the metabolic rate in these experiments decrease to a point where weight gain could be just maintained. It has been reported that adrenocortical extract increases the energy metabolism of both adrenalectomized and thyroidectomized animals (Hoffman, *et al.*, 1948) as well as of normal dogs and mice (Koehler and Hastings, 1929) although the latter point is unsettled. Thus when sesame oil and adrenocortical extract were injected simultaneously the extract maintained the metabolic rate approximately normal, and the animals utilized body reserves so quickly that the liver, damaged by the sesame oil, was unable to keep abreast of energy requirements and thus the animal sustained a loss of weight.

Another possibility is that these relatively large doses of adrenocortical extract caused the observed inhibition of growth as was shown for the rat by Evans, *et al.* (1943). The inhibition of growth

[3] Observations in this laboratory indicate that the livers of rats, frogs and goldfish become rather friable following prolonged periods of injection of sesame oil.

may be partly due to the glycosuria which arises from the increased gluconeogenesis caused by the injected adrenocortical extract (Long, *et al.*, 1940).

Furthermore, it is possible that these doses of adrenocortical extract may cause decreased pituitary activity not only for adrenocorticotrophin but also for somatotrophin, since it has been suggested that testosterone propionate (Rubinstein, *et al.*, 1939) and estrogens (Bugbee and Simonds, 1926) cause a decrease in body weight due to a decrease in somatotrophin production. Finally, there is a possibility that the maintenance of energy metabolism is not due to the adrenocortical extract alone but to some other endocrine gland, such as the thyroid. Verzar (1945) observed a very low calorigenic effect of thyroxine in adrenalectomized animals. Verzar's observations have been substantiated by Hoffman, *et al.* (1940), and since the latter found that adrenocortical extract plus thyroxine had a greater calorigenic effect than either one separately in adrenalectomized and thyroidectomized rats, they suggest that there exists a direct relation between the calorigenic effect of thyroxine and the amount of available adrenocortical hormone. Bock (1940) reports that cortical extract alone does not influence metamorphosis of tadpoles and axolotls, but thyroxin plus cortical extract accelerate metamorphosis greater than thyroxin alone.

SUMMARY

The oxygen consumption of the common goldfish, *Carassius auratus*, was found to be between 0.18 and 0.20 ml. O_2/hr./gm. ("basal") body weight at 25.5°C.

Sesame oil given in relatively large doses (0.0036 ml./gm. of body wt.) every other day for eight weeks was found to decrease the oxygen consumption by *ca.* 28% at the end of the first week. Thereafter, the oxygen consumption fluctuated sinusoidally between various maxima and minima values with time.

Relatively large doses of adrenocortical extract given simultaneously with the sesame oil appear to protect the metabolism against the depressing action of sesame oil. Thus the mean metabolic rates, after a transient increase of *ca.* 13.5% remains at values comparable to those of the controls.

When mean body weights of the respective fish treated with sesame oil are studied, it is found that they show a linear increase with time. Fish treated with sesame oil plus adrenocortical extract, though they fail to show a significant variation when all data are averaged, fall into two component groups: one group gives a mean increase of *ca.* 71% in body weight after eight weeks; the other failed to show any change in body weight.

A number of possibilities which exist to explain the foregoing paradoxical situation have been discussed and further investigations

are being undertaken in order to seek an explanation of the points raised.

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SUCCINIC DEHYDROGENASE AND ANAEROBIC GLYCOLYSIS IN LIVERS OF DIABETIC RATS[1]

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IN THE DIABETIC animal carbohydrate metabolism is markedly altered so that protein and fat storage are decreased (Mirsky, Swadesh and Ransohoff, 1937; Stetten and Boxer, 1944) and a large part of the glucose is lost in the urine, with concomitant loss of depot fat, loss of nitrogen, and failure of growth (Soskin and Levine, 1946; Mackler and Guest, 1949; Young, 1944). In view of the modern concept that enzymes play a fundamental role in cellular metabolism, one can postulate that insulin is interacting with, or in some way affecting the action of enzymes. The results to be described in this report are concerned with the succinic dehydrogenase activity of liver, and with anaerobic glycolysis of liver and brain tissues of alloxan-diabetic lactating rats.

MATERIALS AND METHODS

Lactating female rats three to four months of age, which were primarily used to study the effect of diabetes on lactation and on the enzymes of the corpora lutea were used in the studies. The number of suckling young was reduced to 6 to 8 in all litters.

Diabetes was produced by the intravenous administration of alloxan in doses of 50 mg. per kilogram of body weight, on the 7th or 10th day of lactation. Diabetes was confirmed by blood sugar determinations on mixed blood taken from the cut tip of the tail on the third day following injection of alloxan, and again on the day the rats were used for enzyme studies. The blood glucose determinations were made according to the method of Reinecke (1942).

The animals were killed by cervical dislocation and samples of liver and brain were removed immediately, weighed, and placed in 0.2 ml. of cold glass-distilled water contained in pointed ground glass homogenizing tubes. Glass distilled water was used in making the reagents and the homogenates. The tissue was homogenized and sufficient water added to make a 2.5% homogenate for succinic dehydrogenase determinations and a 5% homogenate for the anaerobic glycolysis studies.

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For succinic dehydrogenase the method of Schneider and Potter was used (1943). Duplicate Warburg flasks were used for each tissue, and 0.2 ml. of the 2.5% homogenate was added to each flask plus sufficient water to make a total of 3 ml. The results are reported in terms of Q_{O_2}, that is, the mm.^{3} of oxygen taken up/mg. of dry tissue/hour. The Q_{O_2} values are averages based on the first four 10 minute periods.

The method of Novikoff, Potter and LePage (1948) was used for determining the anaerobic glycolytic activity of the liver tissue. Slight modifications of this method were found necessary for optimal activity of the tissues used in the present study. Warburg flasks with side arms were used. The flasks contained the following components added in the order cited: Glass distilled water to make a final volume of 3.0 ml., 0.3 ml. of 0.1 M K_2HPO_4-KH_2PO_4 buffer (pH 7.6), 0.3 ml. of 0.5 M $KHCO_3$, 0.1 ml. of 0.01 M K-ATP, 0.3 ml. of 0.2 M nicotinamide, 0.2 ml. of 0.5% K-DPN (added to the side-arm), 0.6 ml. of 0.04 M K-HDP, 0.3 ml. of 0.28 M glucose, 0.1 ml. of 0.1 M $MgCl_2$, 0.1 ml. of 0.15 M K-pyruvate (freshly prepared from 1.0 M pyruvic acid) and 0.3 ml. of 5% tissue homogenate (equivalent to 15 mg. of fresh tissue). All components except the last three were added before the animal was killed and the flasks were kept at refrigerator temperature until ready for use.

After the addition of the homogenate, the flasks were attached to the manometers and gassed from a manifold with 95% N_2:5% CO_2 for 10 minutes. They were then placed in the bath and equilibrated for 5 minutes. The DPN was then tipped in from the side arms. After 5 minutes the zero reading was taken and the measurement of CO_2 evolution was begun. Readings were taken at 10 minute intervals for the next 80 minutes. The $Q_{CO_2}^{N_2}$ values (microliters of CO_2 given off under anaerobic conditions per mg. of dry tissue per hour) were based on the best consecutive sixty minutes (10 to 70 min.).

RESULTS AND DISCUSSION

The data which we have obtained from liver and brain tissue of diabetic lactating rats are summarized in Table 1.

TABLE 1. BLOOD SUGAR AND ENZYMATIC ACTIVITY OF THE LIVER IN LACTATING ALLOXAN-DIABETIC RATS

Day of diabetes	Blood glucose mg./100 ml.	Liver		Brain
		Succinic dehydrogenase Q_{O_2}	Anaerobic glycolysis $Q_{CO_2}^{N_2}$	Anaerobic glycolysis $Q_{CO_2}^{N_2}$
Normal	136 (114–186)	73.0 (6)[1] (69.5–74.9)[2]	37.7 (4) (33.3–39.8)	35.5 (3) (35.2–35.9)
5	495 (463–520)	129.0 (4) (113.3–162.5)	45.2 (4) (42.8–49.7)	34.0 (1)
8	591 (398–920)	146.1 (23) (100.5–218.5)	49.5 (16) (43.0–67.0)	34.0 (5) (32.8–36.3)

[1] No. of rats used.
[2] Range of values obtained.

The results show that after 5 days of diabetes the succinic dehydrogenase Q_{O_2} of liver increased from 73.0 to 129.0, which represents an increase of 77% in the activity as measured by the fortified succinate system. After 8 days of diabetes, succinic dehydrogenase activity increased to a Q_{O_2} of 146.1 which represents 100% increase in activity. There is no overlap of values for succinic dehydrogenase activity in either 5-day or 8-day diabetic animals as compared with controls. There is, however, some overlap in the values obtained for 5-day or 8-day diabetic rats. Other data obtained in this laboratory (unpublished) on male and female diabetic rats show increases in succinic dehydrogenase, so that it cannot be a function of lactation *per se*.

The anaerobic glycolytic $Q^{N_2}_{CO_2}$ of livers from normal lactating rats averaged 37.7, and after 5 days of diabetes the $Q^{N_2}_{CO_2}$ was 45.2, which represents 20% increase in anaerobic glycolytic activity. After 8 days of diabetes the anaerobic glycolytic activity increased further to give an average $Q^{N_2}_{CO_2}$ of 49.5, or 31% increase. At the same time there appeared to be no significant changes in the anaerobic glycolysis of brain tissue. The range of values for anaerobic glycolysis of liver of diabetic rats did not overlap the range of values for normal rat liver, thus adding significance to the increases in enzymatic activity.

The increase in succinic dehydrogenase activity found in diabetes may be postulated to be a direct result of increased production of succinate in the liver although there is no direct evidence available showing this to be true. The evidence that both protein and fat can be metabolized to succinate (Lehninger, 1947; Lorber, Lifson and Wood, 1945), and that both are being catabolized at rapid rates in the diabetic animal and add their intermediates to the metabolic pool (Mackler and Guest, 1949; Soskin, 1941) constitutes the basis for postulating that succinate turnover is increased in diabetic rats. If this is true, then may not the increase in succinic dehydrogenase be an example of enzymatic adaptation such as has been demonstrated in microorganisms (Spiegelman, 1945) and in amphibian embryos (Boell, 1949)? The increase in succinoxidase reported by Tipton, and Nixon (1946) in hyperthyroid rats and by Potter, Price, Miller and Miller (In Press), in the livers of rats fed a non-carcinogenic dye may be additional examples of such adaptation.

The increase in the *in vitro* activity of the glycolytic system of diabetic liver tissue may be a measure of *in vivo* glycolysis or it may be a measure of *in vivo* gluconeogenesis, through reversal of the reactions of glycolysis. The results of Stetten and Boxer (1944) who used deuterium, show that an increased proportion of liver glycogen in diabetic rats is formed from molecules smaller than glucose, and that a portion of the urinary glucose is synthesized from the smaller molecules.

Liver glycogen in the diabetic animal is characteristically low (Soskin and Levine, 1946), but Weber (1946) has reported that after 24 hours fasting the livers of diabetic rats have more glycogen than those of normal rats, and that one hour after glucose feeding, following 24 hours of fasting, the deposition of glycogen is greater in the diabetic than in the normal rat. This suggests an increased rate of glycogenesis in the diabetic animal, which is in agreement with the finding that a greater proportion of glycogen arises from small molecules in the diabetic animal, and with the data we have presented which shows that anaerobic glycolysis is increased in the diabetic rat.

CONCLUSIONS

In diabetic lactating rats the succinic dehydrogenase activity of the liver increases as much as 100% over the activity of livers of normal lactating rats. Smaller increases (up to 31%) occur in anaerobic glycolysis of diabetic rat livers, but not in diabetic rat brain, as compared with normal rat liver and brain. The increases in the activity of succinic dehydrogenase and of the glycolytic enzymes are discussed in relation to enzymatic adaptation mechanisms.

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NOTES AND COMMENTS

EFFECT OF DESOXYCORTICOSTERONE GLYCOSIDE ON THE TUBULAR REABSORPTION OF GLUCOSE[1]

A DIABETOGENIC action has been suggested for desoxycorticosterone acetate. The glycosuria of both the partially depancreatized (Ingle and Thorn, 1941) and the adrenalectomized-phlorhizinized rat (Wells and Kendall, 1940) is increased by DCA. The inactivity of DCA in the glycogen deposition test (Pabst, Sheppard and Kuizenga, 1947) and the increased sensitivity to insulin which follows its administration (Cheng and Sayers, 1949) suggest that desoxycorticosterone does not intervene directly in the intermediary metabolism of glucose but in the mechanism for its renal tubular reabsorption. The following experiments were designed to investigate this possibility.

PROCEDURE AND RESULTS

During studies of the effect of desoxycorticosterone glycoside (DCG) on the electrolyte metabolism of dogs, qualitative tests for glucose were performed on the urine of three animals. The drug was given in doses of 20 mg. per square meter per hour. In each instance sugar appeared in the urine during the period of administration and was absent before and afterward.

In order to determine the mechanism of the glycosuria, clearance studies were performed on a series of four human subjects. Diabetic patients were used to provide conditions analogous to the partially depancreatized animal.

On the assumption that glomerular urine is an ultrafiltrate of plasma, the magnitude of glucose filtration was estimated by the expression:

$$GFR_g = GFR \times P_g$$

where: GFR_g = Rate of glomerular filtration of glucose (mEq./min.);
GFR = Glomerular filtration rate (cc./min.) as measured by the mannitol technique (Goldring and Chasis, 1944).
P_g = Plasma glucose concentration (mEq./cc.).

The influence of tubular activity on glucose excretion was estimated by the expression.

$$TRR_g = GFR_g - U_g V$$

where: TRR_g = Rate of tubular reabsorption of glucose (mEq./min.);
GFR_g = Rate of glomerular filtration of glucose (mEq./min.);
U_g = Urine glucose concentration (mEq./cc.);
V = Urine flow (cc./min.)

The subjects were studied in the fasting state and had received no insulin since the previous morning. Elevation of the blood sugar level to glycosuric levels was insured by intravenous administration of 25 grams of glucose at the beginning of the study and continuous infusion of a 10 per cent glucose solution at the rate of 250 cc. per hour throughout the experimental period. Blood samples were drawn from an antecubital vein. Urine was collected

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through an indwelling soft rubber catheter. The bladder was washed with distilled water after each collection and emptied completely by blowing out with air.

Basal clearance values were determined during two 20 minute control periods following a 20 minute equilibration period. DCG was then administered by continuous intravenous infusion at the rate of 20 mg. per square meter per hour and the clearance values redetermined during three 20 minute test periods.

The effects of DCG were similar in each of the subjects. The mean changes for the group were as follow (Fig. 1):

The plasma glucose level declined slightly during the experiment and averaged 0.0158 mEq./cc. During the control periods glucose was filtered at the rate of 0.592 mEq./min./m.^{2} and reabsorbed at 0.563 mEq./min./m.^{2} As a consequence of this nearly complete reabsorption, the excretion of glucose averaged but 0.029 mEq./min./m.^{2}

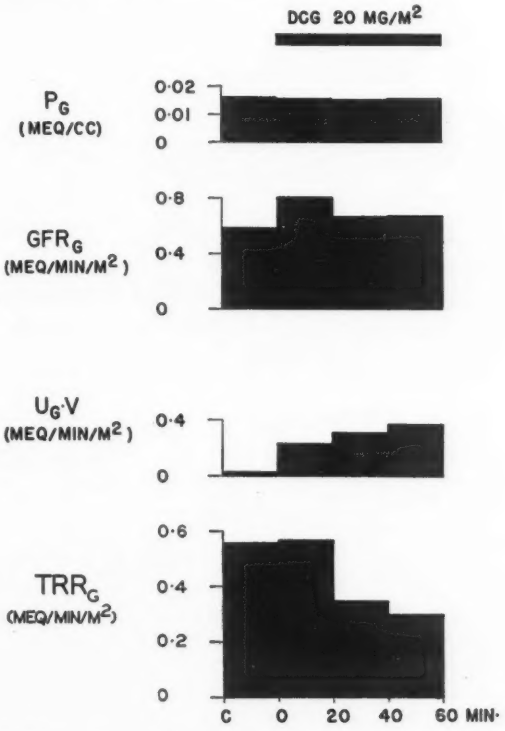

FIG. 1. Mean effects of desoxycorticosterone glycoside on the renal excretion of glucose in four subjects.

P_g = Plasma glucose concentration;
GFR_g = Rate of glomerular filtration of glucose;
U_gV = Rate of urinary glucose excretion;
TRR_g = Rate of tubular reabsorption of glucose.

During the period of DCG administration glucose was filtered at an average rate of 0.719 mEq./min./m.^{2} and reabsorbed at 0.410 mEq./min./m.^{2} Glucose was excreted at the rate of 0.309 mEq./min./m.^{2}, a ten-fold rise over the control value. About 45 per cent of the increase could be accounted for by elevation of the glucose filtration rate; the greater part was due to decrease in tubular reabsorption. By the third test period the rate of reabsorption had fallen to approximately half the control value.

SUMMARY AND CONCLUSIONS

Desoxycorticosterone glycoside was administered to three normal dogs. Sugar appeared in the urine during the interval of DCG administration and was absent in the periods preceding and following the drug.

Clearance studies on four diabetic human subjects indicated that DCG increased glucose excretion principally by decreasing its tubular reabsorption. In view of the glycosuric action of DCA previously reported it appears likely that the mechanism of action involves desoxycorticosterone blockade of the tubular apparatus for glucose reabsorption.

The possibility exists that the glycoside grouping may synergise this action of desoxycorticosterone by providing a phlorhizin-like chemical structure.

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